



Symbiotic Nitrogen Fixation and Nitrate Uptake by the Pea Crop

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SYMBIOTIC NITROGEN FIXATION AND NITRATE UPTAKE BY THE PEA CROP

Erik Steen Jensen

Abstract. Symbiotic nitrogen fixation and nitrate uptake by pea plants (Pisum sativum L.) were studied in field and pot experiments using the ^{15}N isotope dilution technique and spring barley as a non-fixing reference crop. Barley, although not ideal, seemed to be a suitable reference for pea in the ^{15}N -technique. Maximum N_2 fixation activity of 10 kg N fixed per ha per day was reached around the flat pod growth stage, and the activity decreased rapidly during pod-filling. The pea crop fixed between 100 and 250 kg N ha $^{-1}$, corresponding to from 45 to 80% of total crop N. The amount of symbiotically fixed N_2 depended on the climatic conditions in the experimental year, the level of soil mineral N and the pea cultivar. Field-grown pea took up 60 to 70% of the N-fertilizer supplied. The supply of 50 kg $\text{NO}_3\text{-N}$ ha $^{-1}$ inhibited the N_2 fixation approximately 15%. Small amounts of fertilizer N, supplied at sowing (starter-N), slightly stimulated the vegetative growth of pea, but the yields of seed dry matter and protein were not significantly influenced.

(Continued on next page)

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In the present field experiments the environmental conditions, especially the distribution of rainfall during the growth season, seemed to be more important in determining the protein and dry matter yield of the dry pea crop, than the ability of pea to fix nitrogen symbiotically. However, fertilizer N supplied to pot-grown pea plants at the flat pod growth stage or as split applications significantly increased the yield of seed dry matter and protein.

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PREFACE

The present thesis summarizes studies on symbiotic nitrogen (N_2) fixation and nitrate uptake by the pea crop (Pisum sativum L.) using ^{15}N isotope dilution. The ^{15}N dilution technique for the quantification of symbiotic N_2 fixation is critically evaluated.

The work was carried out at the Agricultural Research Department, Risø National Laboratory. I want to thank my 'local' supervisor Arna J. Andersen, Head of the Agricultural Department, and my supervisor at the Department of Soil Fertility and Plant Nutrition and Hydrotechnical Laboratory, The Royal Veterinary and Agricultural University, Copenhagen, Niels Erik Nielsen, for support and advice during the study.

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Finally, I want to express my sincere thanks to colleagues, especially to Jens Sandfær, who promoted these investigations and to L. Henning Sørensen for advice and useful criticism during the preparation of the following papers, which are published or accepted for publication:

- I Jensen, E.S., 1986. Growth and nitrogen fixation in field-grown pea (in press).
- II Jensen, E.S., 1986. Symbiotic N_2 fixation in pea and field bean estimated by ^{15}N fertilizer dilution in field experiments with barley as a reference crop. Plant and Soil 92, 3-13.

- III Jensen, E.S., 1986. The influence of rate and time of nitrate supply on nitrogen fixation and yield in pea (Pisum sativum L.). Fertilizer Research (in press).
- IV Jensen, E.S., A.J. Andersen and J.D. Thomsen, 1985. The influence of seed-borne N in ¹⁵N isotope dilution studies with legumes. Acta Agric. Scand. 35, 438-443.

In the present thesis reference to the four papers will be given by the Roman numerals. The papers I-IV are found in the appendix.

1. INTRODUCTION

The atmosphere above soil contains approximately 79% nitrogen in elemental (N_2) form. Nitrogen in this form is, however, unavailable for plants and other eucaryotic organisms. Some procaryotic organisms, called diazotrophs, have evolved the enzymatic ability to reduce N_2 to ammonia. This process is denoted biological nitrogen fixation (BNF). Diazotrophs may fix nitrogen either independent of other organisms (free-living, asymbiotic), in association with plants which provide the proper environment (e.g. available carbon) for the N_2 fixation process (associative symbiosis) or in a symbiosis where both organisms are interdependent for the process of N_2 fixation (mutualistic symbiosis). The symbiosis between leguminous plants and bacteria belonging to the genus Rhizobium is an example of the latter. Nitrogen is fixed by Rhizobium in nodules on the legume root and made available for synthesis of proteins in the plant. In return the legume delivers the energy in form of photosynthates for the fixation process in the nodules.

Soil nitrogen is primarily in the organic fraction of the soil. This nitrogen came initially from BNF, either asymbiotic or symbiotic. In natural vegetations BNF is still the main source of nitrogen input. In agricultural plant production BNF has been substituted to a still larger degree, especially in the industrialized countries, by industrial fixed N_2 (Haber-Bosch) in synthetic N-fertilizers. However, the world-wide nitrogen input from BNF has been estimated to be four to five times larger than the amount of nitrogen made available as synthetic N-fertilizer (Hardy and Havelka, 1975).

In 1980 24% of the total energy consumption in the Danish agricultural sector was estimated to be associated with the manufacture and use of synthetic nitrogen fertilizers (Hjortshøj Nielsen, 1980). In the production of barley, which is grown on

40% of the cultivated area in Denmark, about 42% of total energy consumption was due to manufacturing and use of synthetic nitrogen fertilizer (Hjortshøj Nielsen, 1980). Although cost of energy for the time being is relatively low, it is likely that cost for production, transport and application of nitrogen fertilizer will continue to rise. Furthermore, the excessive use of nitrogen fertilizer may under some circumstances present a hazard to the environment. Legumes and their symbiosis with Rhizobium offers an alternative or supplement to synthetic N-fertilizer in the production of plant dry matter and protein.

A prerequisite for an increased reliance on symbiotic nitrogen in agricultural plant production is a greater knowledge of 1) the nature of the symbiosis between legume and Rhizobium, 2) the ability of the symbiosis to deliver N for plant growth, 3) how the symbiosis is influenced by environmental factors, and 4) the influence of legume BNF on the environment.

1.1. The pea crop

The pea (Pisum sativum L.), which belongs to the family Papilionaceae (Leguminosae), has been traced to agriculture 7000-6000 B.C. and is now grown world-wide. However, pea is largely confined to temperate regions and the higher altitudes or cooler seasons of warmer regions. In 1981 the world area grown with peas was about 8 mio. ha (FAO, 1981). In Denmark an annual average of about 10000 ha has been grown with peas from 1900 to 1980. Breeding of new cultivars and the EC support policy for protein production have encouraged the farmers to increase the area with pea in Denmark as well as in other Western European countries. Thus, in Denmark the area with pea has increased from about 10000 to 150000 ha from 1980 to 1985 (Danmarks Statistik, 1985).

Pea is cultivated for different uses. The use categories are: dry edible; processing (canning or freezing); fresh; forage and green manure. The dry edible pea constitutes about 90% of peas grown. This crop is harvested at the dry seed stage. Seeds are sold for packeting, for animal feed or for protein and starch extraction. It is this crop which receives much attention in Europe for the time being. The nitrogen nutrition of the dry pea crop is the subject of the present thesis.

1.2. The pea/Rhizobium symbiosis

Leguminous plants are characterized by their high protein content. A well developed pea crop may contain 2 t/ha of protein. Consequently large amounts of nitrogen are needed to obtain high yields. Pea as well as other legumes possess the ability to enter a mutualistic symbiosis with the nodule forming diazotrophe, Rhizobium and fix atmospheric dinitrogen. Symbiotic development and nitrogen metabolism in pea has been reviewed in detail by Pate (1977, 1985). Rhizobia belonging to the cross inoculation group Rhizobium leguminosarum biovar leguminosarum are the microsymbionts in the symbiosis. Efficient R. leguminosarum is present in most cultivated Danish soils (Jensen et al., 1985c), and inoculation with efficient strains is not needed in normal, well-aerated and limed soils (Jensen, 1986a).

The physiology and genetics of the pea/Rhizobium symbiosis have been studied in detail by plant physiologists and microbiologists, e.g. by Pate and co-workers (Belfast), Phillips and co-workers (Davis), Mahon and co-workers (Saskatchewan), Lie and co-workers (Wageningen) and by colleagues at this laboratory. The main part of these studies was carried out under controlled conditions on pot-grown plants. Less information is available about symbiotic nitrogen fixation and combined N uptake by the pea crop under field conditions. This is probably due to the difficulties in measuring the amount of nitrogen fixed in the field.

1.3. Measurement of symbiotic N₂ fixation

In research to improve the efficiency of symbiotic nitrogen fixation by the legume/Rhizobium symbiosis it is essential to be able to quantify how much nitrogen in the plant is derived from the atmosphere. This may either be during a minute, an hour, a day or one or several growth seasons. The fundamental problem in measuring nitrogen fixation is that one must be able to differentiate between nitrogen in the plant derived from the sources: cotyledons, soil, N₂ fixation and under some circumstances fertilizer. The method selected will depend on the objective of the study. The plant physiologists may be interested in measuring diurnal variation in the activity of nitrogenase, whereas the soil scientists may want to quantify the contribution from nitrogen fixation to the soil in a cropping system including legumes. Plant breeders may need selection techniques that are rapid, inexpensive, and non-destructive for screening of large populations for nitrogen fixation capacity.

Nitrogen fixation is measured directly when it is possible to measure some product of the fixation process, and indirectly, when the total N accumulation in the crop is known and the contributions from other N-sources than nitrogen fixation can be measured or estimated. In the following is given a short description of the techniques and their potential application. The first four methods can be classified as direct methods.

Kjeldahl N analysis of the plant material and the subtraction of seed-borne N is an exact estimate of N₂ fixation, only when plants are grown with N₂ fixation as the sole source of nitrogen. The method can be used for quantification of N₂ fixed by plants grown in inactive media (sand, perlite, vermiculite, etc.).

Nitrogen-15-labelled dinitrogen reduction is the most definitive method of quantifying nitrogen fixation. Nodules, excavated nodulated roots or intact roots in closed lysimeters (Sims et

al., 1983; Skøt, 1983) are incubated in an air phase enriched in $^{15}\text{N}_2$. Nitrogen fixation is determined by Kjeldahl and ^{15}N analyses of the plant material. The method is well suited for short-term kinetic measurements of N_2 fixation activity. Due to the cost of $^{15}\text{N}_2$, the volumes needed in lysimeter studies and problems in maintaining a constant enrichment (Sims *et al.*, 1983) the method is not suitable for quantification of the seasonal accumulation of fixed N by a legume crop.

Acetylene (C_2H_2) is reduced to ethylene (C_2H_4) by the nitrogenase of bacteroids (Koch and Evans, 1966). Since this discovery the acetylene reduction assay has been used in many studies. When the 'standard' technique is employed, the nodulated root is incubated in a closed vessel in an air containing acetylene. The amount of ethylene evolved is then measured gaschromatographically after a certain time of incubation. This assay is a short-term kinetic measurement and therefore not suitable for quantification of N_2 fixed over a growth season. This is because of diurnal and seasonal variation in the activity of nitrogenase and because the ratio between acetylene reduced to nitrogen fixed deviates from the theoretical ratio of 3:1 (Skøt, 1983). Furthermore, it is difficult to recover all nodules of field-grown plants. This later problem has been solved by using sealed root chambers (Sims *et al.*, 1983; Denison and Sinclair, 1985). Recently Minchin *et al.* (1983) demonstrated that incubation in acetylene reduces bacteroid respiration of some symbioses. This is another error when using the 'standard' acetylene reduction assay for quantitative purposes. It is possible to overcome this problem by using a flow-through system, where the rate of C_2H_4 production is measured rather than the cumulative concentration as in closed systems (Minchin *et al.*, 1983). The 'standard' acetylene reduction is however suitable for detecting the activity of nitrogenase and for the preliminary screening of legume genotypes or *Rhizobium* strains for enhanced N_2 fixation.

Ureides (allantoin and allantoin acid) are the predominant compounds in which N_2 in some legumes, e.g. soybeans, is transported from nodules to shoot. A correlation between ureid concentration in the plant tissue and the acetylene reduction activity was found by Herridge (1982) and the ureid concentration may be used as an index of N_2 fixation in soybeans (Herridge, 1982; Patterson and LaRue, 1983). The method may be used for screening of soybean material for enhanced N_2 fixation, but is not suitable for quantification of the amount of N_2 fixed over the growth season.

The following two indirect methods and their variants, all have in common that a non-fixing reference crop is needed for the quantification of N_2 fixed by the legume crop.

The difference or N-balance method involves the comparison of total N content of the N_2 fixing crop with a concurrently grown non-fixing reference crop. The total N uptake by the reference crop, which may be a non-fixing legume (uninoculated or non-nodulating isoline) or a non-legume is considered to be an estimate of the soil N contribution to the fixing legume. It is assumed that the legume and the reference crops take up equal amounts of soil N. Fixing and non-fixing crops may either be compared over a single growth season (Witty, 1983; Henson and Heichel, 1984; Rennie, 1984; Jensen et al., 1985a) or over several growth seasons (Lyon and Bizzell, 1934). Stülpnagel (1982) proposed that the measurement of mineral N in soil before and after cultivation of legume and reference and the consideration to a given difference between crops in the amount of residual mineral N, could improve the estimate derived from the difference method. It may be possible, by selection of a suitable reference crop, to obtain reasonable estimates of N_2 fixation by the difference method, when estimating the amount of N_2 fixed at maturity (Witty, 1983; Jensen et al., 1985a).

Nitrogen-15 isotope dilution by $^{14}\text{N}_2$ fixation, is a reliable method for quantifying the amount of N fixed by a legume crop in the field. The legume and a non-fixing reference (uninoculated legume, non-nodulated isoline or non-legume) are grown in plots, where the mineral soil N pool is labelled with ^{15}N . The mineral soil N pool may be labelled by first immobilizing $^{15}\text{NH}_4$ (Legg and Sloger, 1975; Chalk *et al.*, 1983) or incorporating ^{15}N -labelled plant material (Witty and Ritz, 1984; Rennie, 1986) and then await mineralization of the incorporated ^{15}N . The soil N pool may also be labelled by adding either small (McAuliffe *et al.*, 1958; Vallis *et al.*, 1967) or higher (Allos and Bartholomew, 1955; Fried and Broeshart, 1975; 1981) amounts of ^{15}N -labelled nitrate or ammonium. Witty (1983) and Witty and Ritz (1984) also used slow-release formulations of mineral N labelled with ^{15}N . When it is assumed that the same amounts of soil N are available for legume and reference crop and that the reference plant has the same ^{15}N content as the available soil N, it is possible to calculate the amount of N in the legume, which is derived from the air (for calculations see III and IV). This method is suitable for quantifying N_2 fixation up to any point in the life cycle of the plant and evaluating the effect of environment on N_2 fixation in field-grown plants. The main disadvantage of this method is the problem of selecting a proper reference crop (Wagner and Zapata, 1982; Witty, 1983; Ledgard *et al.*, 1985b; I; II).

The natural ^{15}N abundance of the soil N is often found to be higher than of atmospheric dinitrogen. This is probably due to discrimination effects, e.g. during denitrification. These differences in natural ^{15}N abundance have been used for estimating N_2 fixation in legume crops grown together with a non-fixing reference for the estimation of the natural abundance of the soil N pool (Amarger *et al.*, 1979; Kohl *et al.*, 1980). The disadvantage of the method is that the ^{15}N natural abundance of the soil may not be uniform (Rennie *et al.*, 1976). Furthermore, a precise mass spectrometer (double inlet, double collector) is needed for measuring the small differences in ^{15}N natural abundance.

In the present studies (I, II, III, IV) the ^{15}N isotope dilution technique was used for obtaining integrated estimates of N_2 fixed by the pea crop up to different point in the life cycle including the whole growth season and for estimating seasonal rates of N_2 fixation. The mineral soil N pool was labelled with ^{15}N by a single application of ^{15}N -labelled nitrate at seedling emergence. Spring barley (Hordeum vulgare L.) was used as the non-fixing reference crop. The methodology is described in section 2.1 and the method is critically evaluated in section 2.2.

1.4. Objectives

The objectives of the study were:

- to determine the seasonal patterns of growth, symbiotic nitrogen fixation, and mineral N uptake by the pea crop,
- to evaluate the influence of host plant cultivar and growth season on the the total amount of nitrogen fixed by the pea crop,
- to evaluate the effect of small amount of fertilizer N supplied at sowing (starter-N) on the early development, yield and nitrogen fixation and the effect of late applied fertilizer N on the yield of the pea crop harvested at the dry seed stage.

2. NITROGEN-15 ISOTOPE DILUTION

Nitrogen-15 and ^{14}N are stable isotopes of nitrogen with the same chemical properties and only with slight differences in their be-

havior in biological systems. The natural abundance of ^{15}N is 0.366 atom % ^{15}N , and in systems, e.g. soil, where material with a higher concentration of ^{15}N has been added, the change in isotope ratio from the background level permits calculation of the extent to which the tracer has interacted with the system.

The term ^{15}N isotope dilution refers to the decrease in concentration of ^{15}N in a pool by ions or molecules of lower ^{15}N concentration. In the ^{15}N isotope dilution technique for quantifying symbiotic N_2 fixation, the plant N derived from the ^{15}N -enriched mineral soil N pool is diluted by N_2 of natural abundance.

2.1. Methodology

In the present studies the mineral soil N pool in plots with pea and barley (the non-fixing reference crop) was labelled by adding ^{15}N -labelled nitrate to the soil according to the method described by Fried and Broeshart (1975; 1981). Detailed information on the methodology is given by Jensen *et al.* (1985a) and in I-IV. Consequently the following description is given in brief.

Nitrogen-15-labelled (1 to 4 atom % ^{15}N excess) nitrate was sprayed on the soil within a 1 m^2 ^{15}N -microplot within the main plot by the time of seedling emergence. The area surrounding the ^{15}N -microplot was supplied with unlabelled nitrate fertilizer. The N-fertilizer was immediately watered in, and became probably distributed within the upper 0-5 cm of the topsoil.

Plants from the central two rows of the ^{15}N -plot were sampled for ^{15}N -analysis and the remaining plants of the plot were harvested for yield and nitrogen determinations. In the pot experiment (III) the ^{15}N -labelled nitrate was supplied below the soil surface when applied at sowing or at the soil-surface when applied late.

From the isotopic composition of the legume and the reference crop, the N_2 fixation by the legume crop can be estimated. The N_2 fixation may be estimated in different ways, depending on whether different rates of N-fertilizer were used for legume and reference or not. If legume and reference have received different or the same rates of labelled fertilizer, the A-value approach (Fried and Dean, 1952; Fried and Broeshart, 1975; III) can be used. If the legume and the reference are supplied with the same rates of labelled N or the soil N pool has been labelled with ^{15}N in another way than adding readily-available ^{15}N -labelled N-fertilizer as a single dressing at sowing, the approach described by McAuliffe et al. (1958) and Fried and Middelboe (1977) may be used (see IV). The two methods are unifiable if the soil N pool of legume and reference has been labelled in the same way (Fried, 1985) and the difference in the amount of seed-borne N of legume and reference is negligible (IV). The estimate of the proportion of total N derived from nitrogen fixation is yield-independent with both methods of estimation.

In the present study a variation of the method proposed by Fried and Broeshart (1975) was used, since estimates of the amount of soil N in the legume also was of interest. The soil A_N -value was calculated from the ^{15}N -data of the reference crop. The A_N -value for a given nutrient was defined by Fried and Dean (1952) as the amount of available soil nutrient relative to some standard availability associated with the labelled nutrient. Then the amount of N-fertilizer and the recovery of fertilizer in the legume were calculated. An estimate of the soil N uptake in pea was obtained by multiplying the A_N -value with the per cent fertilizer recovery/100. The amount of nitrogen fixed was then calculated by subtracting the amount of N derived from fertilizer, soil and seeds from total crop N. The distribution within the plant of N derived from seed-borne N was determined experimentally for the different crops (IV).

2.2. Evaluation of the method

Several assumptions underlie the estimation of nitrogen fixation:

- When a plant is confronted with two or more sources of nitrogen (e.g. soil and fertilizer), it will take up nitrogen from each source in direct proportion to the amounts available from each source (Fried and Dean, 1952).
- The ratio between assimilated indigenous soil N and added ^{15}N -labelled fertilizer N is the same for the legume and the reference crop. This means that the two crops are able to exploit the same volume of soil for mineral N, due to the same rooting depth and distribution (Fried and Broeshart, 1975).
- The reference and legume crop should have identical patterns of soil N uptake, if the ^{15}N -enrichment of the soil N pool is declining during growth (Witty, 1983). The two crops should at least start to increase in soil N content at the same time and reach maximum soil N content simultaneously (Witty, 1983).
- The effect of added fertilizer N, whether 'real' or 'apparent', on the availability of soil N ('priming', added nitrogen interaction) is negligible or identical for the legume and the reference (Witty, 1983; Jenkinson *et al.*, 1985).
- The difference in the amount of seed-borne N in the legume should be negligible or otherwise accounted for (IV).
- If ^{15}N -analysis is carried out on separate plant parts a weighted atom % ^{15}N excess on a whole-plant basis should be calculated from the ^{15}N -enrichment of individual plant parts. This is because the ^{15}N -enrichment of different plant part may be non-uniform (Fried *et al.*, 1983; Jensen *et al.*, 1985a; II).

- When only a few plants are sampled, the ^{15}N -labelled fertilizer must be distributed evenly over the plot surface. In the present field experiments a frame, separated in 12 squares, was used when the N-fertilizer was applied. This ensured an even distribution.
- The estimate of N_2 fixation is an estimate of the amount of N_2 fixed, which is contained in the crop (e.g. in above-ground plant parts) (Witty, 1983).

Apparently several potentials of errors exist in using this method. The reference crop, which is used for measuring the amount of plant available N in the soil N pool, constitutes the principal potential of error in the ^{15}N technique for quantifying the amount of N_2 fixed. One of the most important factors for obtaining a reliable estimate of N_2 fixation is therefore the matching of legume and reference crop. It should be possible to account for differences in seed-borne N of reference and legume (IV) and for non-uniformity in the ^{15}N -enrichment of different plant parts. However, some of the other assumptions may be only partly fulfilled.

Some studies have indicated that spring barley may not be an ideal reference crop for pea (Witty, 1983; II). The rooting depth of the two crops may differ on clay soils, but not on sandy soils (Vetter and Scharafat, 1964). The experiments reported in appendix II were carried out on sandy clay soils, but the experiment reported in appendix I on a sandy soil. On the clay soil the barley crop may have been able to utilize soil N from greater rooting depth than pea. As a consequence N_2 fixation may have been underestimated (II). In the present studies it was not possible, however, to assess whether the ratios of indigenous soil to added ^{15}N -labelled N assimilated were identical in pea and barley. A method for checking this assumption has recently been published by Ledgard et al. (1985a).

Witty (1983) pointed out that mismatching patterns of soil N uptake in legume and reference crop may lead to error in the estimates of N_2 fixation, when the ^{15}N -enrichment of the soil N pool is declining. This decline occurs when ^{15}N -labelled fertilizer is added at the beginning of the growth season, due to uptake, leaching and immobilization of ^{15}N occurring simultaneously with the mineralization of ^{14}N . The decline in ^{15}N -enrichment can be described mathematically as:

$$\text{atom \% } ^{15}N \text{ in soil} = a + be^{-Dt} \quad (\text{Witty, 1983})$$

where a is the enrichment at time infinity, b is the atom % ^{15}N -excess at time zero, D the decline constant and t the time in days. The faster the decline in ^{15}N -enrichment, the higher is D , and the higher is the potential of error if legume and reference crop have different patterns of N-uptake (Witty, 1983). Witty (1983) found D values between 0.01 and 0.05. From the ^{15}N uptake in barley in the experiment in 1984 (I), the mean enrichment of the soil N taken up between individual harvests was calculated. \log_e to those value were regressed against the time and a D value of 0.040 ($r^2 = 0.996$) was found. A similar value was found in another experiment, where winter rape seed was grown to 'catch' residual ^{15}N -labelled soil N after harvesting pea, supplied with 50 kg N ha⁻¹, at different growth stages. These values indicate that the ^{15}N -enrichment of the soil N pool was declining rather fast. This rapid decline may influence estimates of N_2 fixation only when pea and barley have different patterns of soil N uptake. Pea and barley had rather identical patterns of ^{15}N -fertilizer uptake (I). This indicates that the patterns of nitrogen uptake from the soil N pool are not very different. Furthermore, as also demonstrated by Witty (1983), the influence of mismatching uptake patterns may only be of importance, compared to other sources of error, at low levels of N_2 fixation.

If legume and reference crop are supplied with different rates of ^{15}N -fertilizer it is essential, that the A_N -value is not affected by the rate of N-fertilizer (Fried and Broeshart, 1975). In the study with two rates of N-fertilizer, 25 or 50 kg N ha $^{-1}$ the A_N -values can be deduced from the N uptake by barley at nine harvests (I, Fig. 2). The A_N -value from the first harvest (21 days after emergence) was extremely high due to the low availability of the ^{15}N -fertilizer, which was located in a few cm of the top soil. Five weeks after seedling emergence the A_N -value was estimated to 35 kg N ha $^{-1}$ and was gradually increased to 88 kg N ha $^{-1}$ at maturity. There were only minor differences in A_N -values between N-levels, and the A_N -values were not consistently higher at one N-fertilizer level compared to the other.

If the crops affect the nitrogen immobilization-mineralization processes in soil differently, A_N -values calculated from barley may not be suitable for use with pea. It is not known whether such differences exist, but it was generally found that the maximum ^{15}N -fertilizer recovery was higher in barley than in pea (I, II).

The estimates of N_2 fixed by pea (I, II, III) are estimates of N_2 fixed which is contained in the plant parts harvested. Some of the N_2 fixed by field-grown plants may be excreted from the below-ground plant parts or left in plant residues, which are not sampled. In the field experiments reported in I and II no attempts were made to estimate the amount of N in roots, which was derived from N_2 fixation. However, the root biomass of pea was quantified around flowering in the 1984 experiment (I). About 1.3 to 1.6 t root dry matter per ha was found in the 0-30 cm depth. Pea root dry matter contains 2.0-2.5 % N (III). Roots therefore may contain about 30 kg N ha $^{-1}$ around flowering, of which about 20-30% is derived from fixation (Jensen, unpublished data). The main part of this nitrogen may be translocated to pods during reproductive development, and some lost in decaying nodules and roots. Only a few kilograms of nitrogen per ha is therefore

normally recovered in pea roots at maturity (Jensen *et al.*, 1985a). The amount of N left in root and stubble at maturity may therefore only be negligible at the final harvest. To which extend nitrogen is lost by decay of nodules and exudation of fixed nitrogen is not known. It is well-known, that pea excretes amino acids from the root system (Virtanen, 1938).

It can be concluded that the ^{15}N isotope dilution technique with barley as non-fixing reference crop, although not without problems, seems to be a reliable technique for quantifying the cumulative nitrogen fixation by the pea crop in field. The method might, however, be improved, and it is necessary to stress that several of the assumptions (p. 15-16) underlying the estimates reported in I, II and III, may only be partly fulfilled.

3. SYMBIOTIC N_2 FIXATION AND NITRATE UPTAKE BY PEA

A mature pea crop may contain more than 300 kg N ha^{-1} (I, II). This nitrogen is derived from the seeds sown, from the soil, from symbiotic N_2 fixation and maybe from N-fertilizer depending on the farming praxis. In the following sections (3.1.-3.3.) the results of studies (I, II, III and IV) on role and interaction of these N-sources in the N-nutrition of the pea crop are summarized.

3.1. Growth and nitrogen fixation

In Denmark peas are sown in April with a seeding rate corresponding to 70 to 90 emerged plants per m^2 . This seed may contain from 7 to 10 kg N ha^{-1} ($10 \text{ mg N seed}^{-1}$), which is the first source of N for growth of the germinating seed. This seed-borne N, mainly present in the cotyledons, is evenly distributed between the pea root and shoot during early growth (IV).

Swellings on the primary root, which can be seen a week after seedling emergence, are the first indication of the developing symbiosis. The first white nodules are visible a few days later. Dinitrogen fixing, red nodules will normally be present 10 to 14 days after seedling emergence. More nodules are formed during the following four weeks and plants may have from 50 to 120 nodules, of which 20 to 30 are located on the primary root, by the end of vegetative growth (Jensen et al., 1985c). When flowering is initiated the first formed nodules have already begun to senesce (Pate, 1958).

Pate (1958) reported that pea nodules were fixing N_2 well in advance of exhaustion of the cotyledons for N. This indicates that there is no period of N-deficiency until nodules are formed, if plants are grown without combined N, as it has been suggested by Mahon and Child (1979). Soils of North-Western Europe normally contain significant amounts of mineralized N in spring. This soil N may contribute to the nitrogen nutrition of the pea plant until nodules have developed and are capable of fixing N_2 .

Nitrogen derived from the soil may normally be the principal source of N during vegetative growth (I, II). Symbiotic nitrogen fixation is becoming more significant as N-source in pace with development of the symbiotic system and plants may be able to fix 3 to 4 kg N per ha per day by the end of vegetative growth (I). Even if the first nodules already are senescent the highest rates of N_2 fixation, 10 to 11 kg N ha⁻¹ day⁻¹, are recorded by the end of flowering/early pod development (I). This is also the time of maximum crop growth rate and maximum leaf area index (I).

The rate of N_2 fixation decreases rapidly during the pod-filling growth stages, but some activity is apparently resumed during later growth stages (I). The drop in N_2 fixing activity during pod-filling is simultaneously with a decreasing crop growth rate (I), and plants begin to mobilize nitrogen from vegetative organs to seeds simultaneously with the drop in N_2 fixing activity (I). It was estimated that more than half of total N in pods at maturity was derived from mobilization of N from vegetative organs (I).

Only a small amount of combined N was assimilated during the reproductive growth stages in field-grown pea since the soil N pool was exhausted during vegetative growth. This nitrogen was mainly derived from mineralization of organic bound soil N.

The proportion of total N in 'Bodil' pea, receiving no N-fertilizer, which was derived from N_2 fixation was estimated to vary from 70 to 80% depending on the experimental year and the level of soil N (I, II, III). Since the soil N pool contributed relatively more to the N nutrition during vegetative growth, the contribution from N_2 fixation is normally higher to pods than to vegetative organs (I, II, III).

The plant host cultivar influences the total amount of N_2 fixed during a growth season (II). 'Bodil' pea fixed more nitrogen than 'Timo' pea, but the latter took up more nitrogen from the soil (II). 'Bodil' pea is a dwarf, determinate and white-flowered Danish cultivar, and 'Timo' is a Swedish, tall, indeterminate and purple-violet flowered cultivar. The cultivars accumulated almost the same amount of nitrogen (II), but 'Bodil' was much more efficient in mobilizing N from vegetative organs to seeds (Jensen *et al.*, 1985a), and the seed yield was higher in 'Bodil' than in 'Timo' (II). 'Timo' pea may be deeper rooting than 'Bodil', and consequently have the opportunity to exploit a greater soil volume for N. This higher soil N uptake by 'Timo' may cause the lower N_2 fixation compared to 'Bodil'.

Within each of the cultivars there was a positive correlation between the amount of N_2 fixed and the seed yield during the three experimental years (II). Unfavorable weather conditions during the periods of pod-filling and maturity were the cause of the low yields and low N_2 fixation in 1980. In 1981 climatic conditions were better for pea cultivation, but there was also much rain during the period of maturity. In 1982 growth conditions were excellent, with rainfall during early vegetative growth and around flowering and high temperatures and dry

conditions during the period of maturation (Jensen *et al.*, 1985a). In 1984 the climatic conditions for cultivation of pea were also rather good, which also was reflected in the high yields and high amounts of N_2 fixed (I). The different seed yields and amount of nitrogen fixed within the three years, indicate that environmental conditions may be more important than the ability to fix N_2 in determining the yield of seed dry matter and protein of the pea crop. The different yields obtained also highlighted the role of nitrogen mobilization from vegetative organs to pods in determining the final seed yield. In 1980 where low yields were obtained, the amount of N left in vegetative plant residues was almost twice that left in residues in 1982 and 1984, where the pea seed yield was about 7 t ha^{-1} .

3.2. Nitrate uptake, inhibition of N_2 fixation and the effect of nitrate on the yield

The pea crop was almost as efficient as barley in recovering the ^{15}N -labelled nitrate (I, II, III). At maturity 64 and 70% of the 50 kg N ha^{-1} applications were recovered in 'Bodil' pea and barley, respectively (I, II). There was, however, a tendency, that the recovery of fertilizer N in barley was higher during earlier growth stages (I, II). The net loss of ^{15}N -labelled fertilizer apparently was higher in barley than in pea during the late growth stages.

The patterns of ^{15}N -nitrate uptake in pea and barley, however, were slightly different, especially during the early growth stages, where barley took up nitrate faster than pea. The patterns of nitrate uptake in field-grown pea were slightly different in the different studies. In 1984 the maximum ^{15}N fertilizer content was reached around the full bloom/flat pod growth stage (I), but in 1980 and 1981 the crop was still taking up fertilizer N during the pod-filling stages (II).

Nitrate applied around the flat pod growth stage was recovered more efficiently than nitrate applied at sowing (III). Presumably, this was because the later the N was supplied the higher was the growth rate, the quicker was the uptake and the less was the immobilization of fertilizer N (III).

The well-known inhibition of N_2 fixation by nitrate depends on the soil concentration of nitrate. In the field experiments the 50 kg N ha^{-1} application inhibited the total N_2 amount of N fixed with about 15% (I, II), but the inhibition seemed only to occur during the stages of growth with high rates of nitrate uptake (I). The supply of 25 kg N ha^{-1} did not significantly influence the amount of N_2 fixed at maturity (I). Small amounts of nitrate N supplied at sowing neither influenced the N_2 fixation of pot-grown plants, but high amounts significantly inhibited the N_2 fixation (III).

One of the reasons for supplying legumes with fertilizer nitrogen is that seedlings may suffer from N-deficiency if mineral is not present in the period until functioning nodules are formed (Mahon and Child, 1979). In their experiment they found a stimulation of the dry matter production, when nodulated pea plants, grown in an N-free medium, were supplied with ammoniumnitrate at sowing (starter-N). The idea of supplying legumes with starter-N is that the mineral N should stimulate early vegetative development, which would result in a greater capacity for N_2 fixation when the combined N was assimilated (Mahon and Child, 1979).

The present experiments (I, II) were carried out on soil containing from 30 to 60 kg nitrate and ammonium N in the plough-layer. There were only slight effects of starter-N on the early growth of pea (I, II). Neither was there any effect on the seed yield (I, II). Starter-N neither influenced the seed yield significantly in the pot experiment, but the dry matter yield of vegetative organs was slightly increased (III). The soil N, derived from mineralization of organic bound N, which is present in the soil

in spring, seems to be sufficient in supporting N-nutrition of the pea crop until the symbiotic system takes over the nitrogen nutrition of the crop. This conclusion was also reached by other authors (Mulder, 1948; Cutcliffe and Munro, 1979; Augustinussen, 1986).

The rate of N_2 fixation is decreasing during early pod development (I). This is a growth stage with high nitrogen demands of the seeds. Supplying pot-grown plants with nitrate at this growth stage, significantly increased the yield of seed dry matter and nitrogen (III). This may be due to a delay in the remobilization of N from vegetative organs, and consequently a delay in the hydrolysis of functional leaf proteins, whereby photosynthesis is prolonged. This effect of late applied nitrate on the yield of pea has yet to be demonstrated in the field.

Supplying pot-grown peas with very high amount of nitrate at sowing adversely affected the yield (Andersen *et al.*, 1983). This may have been due to salt effects, or due to an effect of increased pH, caused by nitrate assimilation, on the availability of other nutrients. Splitting the N-fertilizer in three or four applications resulted in a total dry matter production, which was 20 to 40% higher than in the pea receiving no N-fertilizer, and higher than all other N-fertilizer treatments (Andersen *et al.*, 1983; III).

3.3. The influence of symbiotic N_2 fixation by pea in a cropping system

Symbiotic nitrogen fixation accounts for 80 to 90 % of the nitrogen removed in seeds by the pea crop (I, II). This indicates that the cultivation of a grain legume like pea will not result in an improved soil N status. However, since more nitrogen will be left in plant residues from a pea crop (50 to 100 kg N ha⁻¹) than in residues from a cereal crop (e.g. 30 kg N ha⁻¹ from barley), the growing of pea may result in a slower decrease in the total amount of soil nitrogen compared to the growing of a cereal.

Pea nodules and roots are decomposed very quickly during the late growth stages and during autumn. Pea straw will also decompose during autumn, but the degree to which the decomposition will result in a net mineralization of nitrogen is highly dependent on the C/N ratio of the material (L. H. Sørensen, unpublished data). It has been observed (Haahr et al., 1985), that the soil profile contains more mineral N after the harvest of a pea crop than after oats. It is therefore important, that a crop is present during the autumn to take up this nitrogen. Otherwise it will be leached.

Studies on the residual effect of pea compared to oats (Haahr et al., 1985), indicated that the residual effect in a following spring barley crop was comparable to approximately a saving of 15 kg N ha⁻¹ in fertilizer compared to oats. The residual effect in winter barley or winter wheat following pea was about 30 to 35 kg N ha⁻¹ compared to oats. Presumably, the main value of pea in the crop rotation is long-term. This means that the residues with a high nitrogen concentration contribute to maintaining soil organic N content to ensure delivery of N to future crop. However, more information is needed concerning the fate of nitrogen left in the residues from the pea crop.

4. FUTURE OUTLOOK

4.1. Symbiotic nitrogen fixation by pea

Symbiotic N₂ fixation may be a factor in the improvement of pea as a protein crop. However, other factors, e.g. agronomic, are probably more important than the capacity (kg N fixed) and efficiency (kg C used per kg N fixed) of symbiotic N₂ fixation in the improvement of pea as a protein crop. One of these factors is the standing ability, which have been improved by development of new plant ideotypes, e.g. the 'semi-leafless' cultivars (Snoad, 1985; Borgefors et al., 1986).

However, genetic variations in the capacity and efficiency of symbiotic nitrogen fixation have been demonstrated within most legume species and Rhizobium cross inoculation groups. Breeding for enhanced N_2 fixation has also been successful in some legume species, e.g. alfalfa (Barnes et al., 1984). Gelin and Blixt (1964) demonstrated that the seed yield is positively correlated with the number of root nodules formed, and Hoobs and Mahon (1982a, b) found immense variation in the N_2 fixation activity and physiological characters associated with N_2 fixation within Pisum sativum. This indicates that it may be possible to improve pea N_2 fixation by breeding. For the time being classical plant genetics may be more efficient in doing this than plant molecular biology (Brewin et al., 1985).

Differences exist within the pea/R. leguminosarum symbiosis with respect to how much carbohydrate is consumed by nodules in nitrogen fixation (Witty et al., 1983). As an average the costs have been estimated to 11 kg carbohydrate per kg N fixed (Mahon, 1983), which means that 2.2 t carbohydrate may be consumed by the nodules in the fixation of 200 kg N by a pea crop. Little information is available about the ability of the crop to compensate for such carbon cost via increased photosynthesis. If it is assumed that the crop only partly will be able to compensate for these costs, perhaps it is possible to improve the dry matter yield of pea by improving the carbon economy of the fixation process in the nodules by selection or genetic manipulation of the Rhizobium strains. However, such improvements require 1) information on which characters in Rhizobium are associated with the higher efficiency in carbohydrate utilization in the symbioses, and 2) that the manipulated strains are more competitive for nodule formation than the indigenous strains in soil, when inoculated in the field. Another approach would be to select pea genotypes which has a preference for nodulation by the highly efficient strains of the mixed indigenous populations.

It is well-known that the dry matter production of nitrate-grown legumes is higher than of symbiotically grown plants under controlled growth conditions. In fields harbouring efficient strains of Rhizobium leguminosarum an assessment of the possibilities for improving the yield by reducing the costs of N_2 fixation should be the comparison of an N_2 fixing crop with a crop receiving non-limiting amounts of nitrate split over the growth season. The pot experiment reported in (III) indicated, that when the nitrate fertilizer was supplied as split (three) applications the dry matter yield was significantly increased compared to symbiotically grown non-fertilized peas and to peas receiving the same amount of nitrate as the total of the split applications, but as one application at sowing.

In the present study it was demonstrated that the N_2 fixation activity is decreased during pod-filling, which is a growth stage where the pea pods have high requirements for nitrogen. Consequently, N is mobilized from the vegetative organs to pods. This leads to the so-called 'self-destruction' of the plant, since the photosynthetic apparatus is destroyed. If the period of high rates of N_2 fixation could be prolonged, it was perhaps possible to delay the destruction of the photosynthetic apparatus. In this way the period of active photosynthesis could be prolonged, which again could lead to higher yields and higher amounts of N_2 fixed. Differences in the pattern of N_2 fixation (acetylene reduction) during pod-filling has been demonstrated within pea (Bingefors et al., 1986). Prolonged photosynthesis and N_2 fixation will probably also be associated with later maturity.

Although the pea/R. leguminosarum seems to be rather tolerant to nitrate, the establishment of the symbiosis may be impaired or delayed, when considerable amounts of mineral nitrogen are present in the soil in spring. Tolerance of the symbiosis to nitrate perhaps could improve the N-status of the pea crop through the complementary assimilation of N_2 and NO_3^- . It has been demonstrated that the pea cultivar may influence the short-term effect of nitrate on nodule growth and nitrogenase activity (Jensen, 1986b).

The pea cultivars 'Bodil' and 'Timo' seem to differ in their ability to take up soil mineral N (II). It is important that the ability of the pea crop to take up soil mineral N is not impaired concurrently with an improved ability to fix nitrogen symbiotically. It is well-known that the dwarf pea cultivars also have smaller root systems. If pea is not exhausting the soil efficiently for nitrate, increasing the area with peas may result in the environmental problems associated with leaching of nitrate, if the pea crop is not followed immediately by an under-sown or following crop. Simultaneously with breeding for improved nitrogen fixation it is important to consider the ability of the pea crop to utilize available soil mineral N.

If pea is grown in intercrop with a cereal the soil mineral N probably will be utilized efficiently. Studies of the N-utilization in mixtures of pea and barley have indicated, that barley is much more competitive for soil mineral N than pea and that the mixtures took up applied ¹⁵N-fertilizer as efficient as barley grown in pure stand (Jensen et al., 1985b). The amount of symbiotically fixed N per plant was lower in the mixtures than in the pure stand, probably due to competition for other growth factors, e.g. light (Jensen et al., 1985b). Breeding of peas suited for intercropping with cereals probably should concentrate on the influence of competition on N₂ fixation. Increasing the ability of pea to fix nitrogen in intercrops with cereals probably will increase the advantage from growing such crops.

As it was indicated in section 3.3. the soil may contain more available nitrogen after a pea crop than after a cereal. This may be due to the early decomposition of nitrogen rich plant residues. Future studies should be addressed to the management of symbiotically fixed nitrogen in cropping systems to optimize its utilization by the legume itself and its benefit to non-legume crops in the cropping system. For the legume this would imply an improvement of the nitrogen harvest index (for the plant parts harvested). For the other crops in the cropping system this would require design of crop systems without fallow periods with the potential of leaching of nitrogen derived from mineralization of nitrogen left in residues from previous legume crops.

There seems to be several possibilities to improve the contribution from symbiotic nitrogen in pea to dry matter and protein production in cropping systems. Whether this improvement will be achieved quicker by a more direct effort on breeding for enhanced N_2 fixation, or the improved capability for N_2 fixation will be carried along with the selection process for improved yield by the legumes, as indicated by Coale *et al.* (1985), e.g. by a better standing ability of the pea crop, is difficult to say.

4.2. Improvement of the methodology for quantification of nitrogen fixation by the legume/Rhizobium symbiosis

The methodology selected for measuring N_2 fixation will depend on the nature of the study. The ^{15}N isotope dilution technique may be of interest to crop physiology studies. When used in the field the technique can be improved in several ways.

Firstly, a detailed study of the best reference crop for a given legume would be valuable. The method proposed by Ledgard *et al.* (1985a) may be used for checking that the ratio between the indigenous soil N and the added ^{15}N fertilizer N assimilated is identical for legume and reference. Secondly, a stabilization of the ^{15}N -enrichment of the soil N pool would make the selection of the reference crop less critical. The ^{15}N -enrichment may be stabilized by using slow-release fertilizer (Witty, 1983), by adding ^{15}N -labelled organic material (Witty and Ritz, 1984; Chalk *et al.*, 1983; Rennie, 1986) or by immobilizing $^{15}NH_4$ with available carbon prior to the experiment (Legg and Sloger, 1975). Soils previously supplied with ^{15}N would presumably also have a more stable enrichment of the soil N pool during the growth season (Kohl and Shearer, 1981).

In a field experiment milled pea seed and straw and barley straw labelled with ^{15}N (approx. 1 atom % excess) was added at a rate of about 100 kg N ha^{-1} and mixed within the soil in the autumn.

The following year the soil was cropped with pea and barley and three harvests were taken to determine the ^{15}N -enrichments of the plant material and to estimate N_2 fixation by pea. The ^{15}N -enrichment in barley was 0.24, 0.20 and 0.20 atom % ^{15}N excess 36, 59 and 98 days after seedling emergence, respectively (Jensen, unpublished data). In the experiment reported in paper I, barley supplied with 50 kg N ha^{-1} and harvested at approximately the same dates, had ^{15}N -enrichments of 0.58, 0.50 and 0.37 atom % excess, respectively. This indicates the stabilizing effect of adding the ^{15}N on organic form. The ^{15}N -enrichment is stabilized, because the indigenous non-labelled N and the newly added ^{15}N -labelled N is mineralized at approximately the same rate. The proportion of total N derived from fixation in the pea cultivar 'Frisson' was estimated to 42, 80 and 80 %, 36, 59 and 98 days after emergence, respectively (Jensen, unpublished data). In this connection one should address the importance of preserving ^{15}N -labelled plant material, since it may be valuable for new experiments.

The difference method for estimating the amount of N fixed by a pea crop at maturity was compared to the ^{15}N dilution method by Jensen et al. (1985a). It was concluded that it is possible to obtain reasonable estimates by this method, provided a suitable reference crop has been chosen. In the present experiments (I, II, III) barley recovered almost the same amount of fertilizer N as pea, and as a consequence the difference method should give reasonable estimates. In the study reported in I the ^{15}N isotope dilution based estimate of N_2 fixation was 232 kg N ha^{-1} as mean of N-levels. Using the difference method and accounting for the seed-borne N N_2 fixation was estimated to 236 kg N ha^{-1} . In studies of the seasonal pattern of N_2 fixation by pea, the difference method is unsuitable, because the patterns of soil N uptake in pea and barley may not be identical.

In plant breeding programmes some quick non-destructive technique is essential for screening large populations for N_2 fixation activity. Characters associated with N_2 fixation, e.g., nodule

mass or acetylene reduction activity, may be used in early selection procedures (Heichel et al., 1985; Mytton and Rys, 1985). During later selection procedures the difference method with a proper reference crop may be employed in the field. The ^{15}N dilution technique may be used to screen for high N_2 fixation capability on soil lightly enriched in ^{15}N . In breeding programs having the objective of improving the N_2 fixation, it may only be necessary to determine the ^{15}N -enrichment of some plant part and not necessarily use a reference crop, since comparisons among genotypes may be of primary interest.

5. SUMMARY

Pea harvested at the dry seed stage is becoming of increasing importance as a protein crop in Europe. The pea crop is normally not supplied with fertilizer nitrogen, because the pea like other legumes are able to fix dinitrogen (N_2) symbiotically in root nodules formed by Rhizobium bacteria. The yield of the pea crop is therefore dependent on the ability of the symbiosis to support growth of the plant. Symbiotic nitrogen fixation by the plant/Rhizobium symbiosis may become more important in future agricultural plant production. A prerequisite for an increased reliance on biologically fixed nitrogen in agricultural plant production is a greater knowledge of 1) the nature of the symbiosis between the host plant and Rhizobium, 2) the ability of the symbiosis to deliver N for plant growth, 3) how environmental factors influence the fixation of dinitrogen, and 4) the influence of legume BNF on the environment.

In studies of symbiotic nitrogen fixation it is important to be able to measure the rate and/or the amount of nitrogen fixed. In section 1.3. is given a brief review of existing methodology for measuring symbiotic nitrogen fixation.

The aim of the present investigations was to study the symbiotic nitrogen fixation and nitrate uptake by pea (Pisum sativum L.) in field and pot experiments by the use of the ^{15}N isotope dilution technique. ^{15}N -labelled nitrate was supplied as a single application at seedling emergence in the field experiments. Spring barley was grown as the non-fixing reference crop to determine plant available soil N. Using this method it is possible to obtain an integrated estimate of N_2 fixed up to any point in the life cycle of the crop. The selection of the reference crop has been reported to constitute the principal potential of error in the method. Barley, although not ideal, seemed to be a suitable reference for pea (Section 2.2.).

The results of the studies can briefly be summarized as follows:

The rate of symbiotic nitrogen fixation increased in parallel with the crop growth rate during vegetative growth and reached a maximum of 10 kg N fixed per ha per day at the flat pod growth stage. During the following weeks of pod-filling the rate of N_2 fixation rapidly declined, but some activity was apparently resumed during later growth stages (Section 3.1., I). The decline in the N_2 fixing activity was simultaneously with lodging of the crop, decreased crop growth rate, decreased green leaf area index and the initiation of mobilization of N from vegetative organs to pods. It was estimated that more than half of total N present in pods at maturity was derived from mobilization of N from vegetative organs (Section 3.1., I).

The total amount of nitrogen fixed during a growth season varied between 100 and 250 kg N per ha, depending on the climatic conditions of the experimental year, the level of soil mineral N and the pea cultivar. These amounts of N_2 fixed corresponded to from 45 to 80% of total above-ground crop nitrogen (Section 3.1., I, II).

The white-flowered and dwarf pea cultivar 'Bodil', which is commonly grown in Denmark, took up the applied fertilizer N almost as efficiently as barley (60 to 70%). N_2 fixation in field was inhibited about 15%, when the crop was supplied with 50 kg N ha^{-1} . This inhibition occurred mainly during that period of vegetative growth, where the nitrate fertilizer was taken up and shortly after. Low amounts of nitrate supplied at sowing did not significantly influence the nitrogen fixation in field or pot grown plants (Section 3.2., I, II, III).

Small amounts of nitrate fertilizer supplied at sowing or seedling emergence (starter-N) slightly stimulated the early growth of pea, but had no significant effect on the yield of seed dry matter and nitrogen. The available soil nitrogen and symbiotic nitrogen seemed to be able to support the growth of pea for the obtainment of high yields. In the present field experiments environmental conditions seemed to be more important than the ability to fix nitrogen symbiotically in determining the dry matter and protein yield of pea.

However, nitrate supplied at the flat pod growth stage or supplied as split applications to pot-grown pea plants significantly increased the yield of seed dry matter and protein, indicating that it may be possible to improve the nitrogen nutrition of the pea crop. (Section 3.2., I, II, III).

The role of symbiotic nitrogen fixation by pea in a cropping system is discussed in section 3.3. and future prospects of research on symbiotic nitrogen fixation by pea and possible improvements of the ^{15}N -methodology for measuring symbiotic nitrogen fixation by legumes are discussed in section 4.

6. SAMMENDRAG

Denne afhandling er et sammendrag af fire artikler omhandlende undersøgelser over symbiotisk kvælstofbinding og nitratoptagelse hos ært dyrket til modenhed. Tre af artiklerne er publiceret og den tredje er under forberedelse for publicering. Artiklerne er trykt i appendixet.

Ærter dyrket til modenhed er ved at få større betydning som proteinafgrøde i Europa. Ærteafgrøden tilføres normalt ikke kvælstofgødning, da ært i lighed med andre bælplanter er i stand til at binde atmosfærisk kvælstof (N_2) i symbiose med rodknoldbakterien Rhizobium. Udbyttet af ærteafgrøden er derfor afhængigt af symbiosens evne til at levere kvælstof til plantevækst. Biologisk kvælstofbinding i plante/Rhizobium symbiosen kan blive af stor betydning i landbrugets fremtidige planteproduktion. En forudsætning for, at den biologiske kvælstofbinding kan anvendes i et større omfang, er, at der opnås et bedre kendskab til 1) vært-plante/Rhizobium symbiosens natur 2) den symbiotiske kvælstofbindings evne til at forsyne værtplanten med kvælstof, 3) hvorledes miljøfaktorer indvirker på bindingen af atmosfærisk kvælstof, samt 4) hvorledes bælplanternes kvælstofbinding indvirker på miljøet.

I studier af den symbiotiske kvælstofbinding er det desuden vigtigt, at man er i stand til at måle kvælstofbindingsrater samt hvor meget kvælstof, der totalt kan bindes af planten. I det indledende afsnit 1.3. er der givet en kort oversigt over eksisterende metodik til bestemmelse af symbiotisk kvælstofbinding.

Formålet med disse undersøgelser var at belyse størrelsen af den symbiotiske kvælstofbinding og nitratoptagelsen hos ært (Pisum sativum L.) i mark- og karforsøg ved anvendelse af ^{15}N isotop fortynding. ^{15}N -beriget nitrat blev tilført ved fremspiring i markforsøgene. Vårbyg blev dyrket som den ikke-kvælstofbindende

referenceafgrøde. Ved anvendelse af denne metode er det muligt at opnå integrerede estimater af kvælstofbindingens størrelse frem til ethvert tidspunkt i afgrødens udvikling. Valget af referenceafgrøde synes i følge andre undersøgelser at udgøre den største risiko for en fejlagtig bestemmelse af kvælstofbindingen. Vårbyg var ikke en ideel reference, men synes dog at være anvendelig sammen med ært (afsnit 2.2.).

Resultaterne af undersøgelserne kan kort resumeres således:

Kvælstofbindingsraten hos ært forøgedes parallelt med afgrødens vækstrate under den vegetative vækstfase og nåede et maximum på ca. 10 kg N fikseret per ha per dag ved 'flad bæg' vækststadiet. I de følgende uger, hvor bælgene blev fyldt, reduceredes kvælstofbindingsraten, men nogen aktivitet kunne tilsyneladende konstateres ved senere vækststadier (afsnit 3.1., I). Kvælstofbindingsraten blev reduceret samtidigt med at afgrøden gik i leje, afgrødens vækstrate blev reduceret, og der var en reduktion i afgrødens grønne bladareal index. Desuden påbegyndtes mobiliseringen af kvælstof fra de vegetative plantedele til bælg. Det blev beregnet, at mere end halvdelen af det totale kvælstofindhold i bælgene ved modenhed hidrørte fra mobilisering af N fra de vegetative plantedele (afsnit 3.1., I).

Den totale mængde kvælstof, som blev bundet i løbet af en vækstsæson, varierede mellem 100 og 250 kg N per ha og afhang navnlig af de klimatiske betingelser i vækstsæsonen det pågældende forsøgsår, niveauet af plantetilgængeligt kvælstof i jorden samt af ærtesorten. De pågældende mængder symbiotisk bundet kvælstof svarede til fra 45 til 80% af det totale kvælstofindhold i afgrødens overjordiske plantedele (afsnit 3.1., I, II).

Den hvid-blomstrede lave ærtesort, 'Bodil', som er almindeligt dyrket i Danmark, var i stand til at optage den tilførte kvælstofgødning omtrent lige så effektivt som vårbyg (60 til 70% optagelse). Kvælstofbindingen i marken blev reduceret ca. 15% ved

tilførsel af 50 kg N per ha. Denne reduktion af kvælstofbindingen fandt hovedsageligt sted i den periode af den vegetative vækst, hvor optagelsen af kvælstofgødningen fandt sted. Mindre mængder nitrat tilført ved såning havde ikke signifikant indflydelse på kvælstofbindingen hos ært (afsnit 3.2., I, II, III).

Mindre mængder nitrat-gødning tilført ved såning eller fremspiring (start-kvælstof) havde en svagt stimulerende effekt på den tidlige vækst af ært, men ingen signifikant indflydelse på udbyttet af frøtørstof og -protein. Det plantetilgængelige jordkvælstof og kvælstof hidrørende fra den symbiotiske kvælstofbinding var tilsyneladende i stand til at dække ærteafgrødens kvælstofbehov for opnåelse af høje udbytter. I nærværende markforsøg synes miljøfaktorer, især fordelingen af nedbøren over vækstsæsonen, at være af større betydning for opnåelse af et højt udbytte af frøtørstof og -protein hos ært end evnen til at binde kvælstof i symbiose med Rhizobium. (afsnit 3.2., I, II, III).

Nitrat tilført ved 'flad bælg' stadiet eller tilført delt til ærteplanter i kar forøgede imidlertid udbyttet af frøtørstof og -protein signifikant. Dette antyder, at det er muligt at forbedre kvælstofforsyningen af ært (afsnit 3.2., I, II, III).

Betydningen af ærts symbiotiske kvælstofbinding i sædskiftet er diskuteret i afsnit 3.3, og perspektiver for yderligere undersøgelser over kvælstofbindingen hos ært samt mulighederne for at forbedre ¹⁵N-metodiken til bestemmelse af kvælstofbinding hos bælgplanter er diskuteret i afsnit 4.

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APPENDIX

ARTICLES I - IV

Growth and nitrogen fixation in field grown pea

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Key words Assimilate partitioning Growth analysis Leaf area
Nitrate Nitrogen fixation ^{15}N isotope dilution Pea Pisum
sativum

Summary The seasonal patterns of growth and symbiotic N_2 fixation under field conditions were studied by growth analysis and use of ^{15}N -labelled fertilizer in a determinate pea cultivar (Pisum sativum L.) grown for harvest at the dry seed stage.

The patterns of fertilizer N-uptake were almost identical in pea and barley (the non-fixing reference crop), but more fertilizer-N was recovered in barley than in pea. The estimated rate of N_2 fixation in pea gradually increased during the pre-flowering and flowering growth stages and reached a maximum of 10 kg N fixed per ha per day nine to ten weeks after seedling emergence. This was the time of early pod-development (flat pod growth stage) and also the time for maximum crop growth rate and maximum green leaf area index. A steep drop in N_2 fixation rate occurred during the following week. This drop was simultaneous with lodging of the crop, pod-filling (round pod growth stage) and the initiation of mobilization of nitrogen from vegetative organs. The application of fertilizer-N inhibited the rate of N_2 fixation only during that period of growth, when the main part of fertilizer-N was taken up and shortly after. Total accumulation of fixed nitrogen was estimated to be 244, 238 and 213 kg N ha $^{-1}$ in pea supplied with nil, 25 or 50 kg NO_3^- -N ha $^{-1}$, respectively.

About one-fourth of total N_2 fixation was carried out during pre-flowering, one-fourth during the two weeks of flowering and the remainder during post-flowering. About 55% of the amount of N present in pods at maturity was estimated to be derived from mobilization of N from vegetative organs. "Starter" N (25 or 50 kg NO_3^- -N ha $^{-1}$) did not significantly influence either dry matter and nitrogen accumulation or the development of leaf area. Neither root length and root biomass determined 8 weeks after seedling emergence nor the yield of seed dry matter and nitrogen at maturity were influenced by fertilizer application.

Introduction

Symbiotic N_2 fixation in field-grown legumes is influenced by many environmental factors (Sprent and Minchin, 1983). Combined N is known to inhibit N_2 fixation when present at high levels in the soil (Oghoghorie and Pate, 1971; Jensen, 1986b). On the other hand, a certain level of combined N may be needed during early crop development in order to overcome N-deficiency during the period from when the cotyledon N-sources are exhausted until nodules are formed and capable of supplying the plant with symbiotically fixed nitrogen (Mahon and Child, 1979). However, it has been observed that temperate legumes, like pea, in contrast to tropical legume species, do not suffer from a period of nitrogen hunger following the exhaustion of cotyledons (Pate, 1958; Sprent and Minchin, 1983).

During later growth stages in pea and other legumes N_2 fixation may be the only N-source for growth. However, studies using the "standard" acetylene reduction (AR) assay (incubation in closed vessels), have demonstrated that AR-activity reaches a maximum around flowering and is reduced during fruit/seed formation (LaRue and Kurz, 1973; Lawrie and Wheeler, 1973; Bethlenfalvay and Phillips, 1977, Bethlenfalvay et al., 1977; Dear and Clark, 1980; Young, 1982). It has also been observed that more than 50% of the pea root nodules may be destroyed before flowering (Pate, 1958). This indicates that a high proportion of the N requirements of seeds is derived from mobilization of nitrogen from vegetative organs. Pate (1985) recently reported that in a determinate cultivar only about 35% of total pod nitrogen is assimilated post-flowering.

The "standard" AR-technique, which has been used for establishing profiles of N_2 -(C_2H_2)-reduction during growth (e.g. LaRue and Kurz, 1973), cannot be used for quantification of N_2 fixation, because it is extremely difficult to get a sound estimate of the amount of nodule tissue in field-grown plants and because the ratio of C_2H_2 -reduction to N_2 fixation often deviates from the theoretical ratio of three. Furthermore, it has recently been demonstrated that the "standard" AR-assay may be erroneous, due to the effect of acetylene on bacteroid respiration (Minchin *et al.*, 1983). The ^{15}N isotope dilution technique, even though it is not without problems, is the most reliable method for quantifying N_2 fixation. However, in most studies, when this technique has been used, only a single determination of N_2 fixation was made at maturity (Witty, 1983).

The aims of the present study were 1) to determine the course of N_2 fixation in field-grown pea, and 2) to evaluate the effects of "starter" N on growth and N_2 fixation in pea. Growth analysis and the ^{15}N dilution technique with barley as reference crop were used.

Methods

Site

The experiment was carried out in 1984 on a loamy sand soil in Risø experimental fields. The field was cropped with spring barley in 1983 and 30 kg P and 50 kg K ha^{-1} were applied before sowing in 1984. The soil contained 30 kg $NO_3^- + NH_4^+$ -N ha^{-1} at sowing in the 0-25 cm layer; pH was 6.9 and the cation exchange capacity 15 meq 100 (g soil) $^{-1}$.

Experimental design

Field pea (*Pisum sativum* L.) 'Bodil', an early, white-flowered, dwarf and determinate cultivar was used. Nil, 25 or 50 kg NO_3 -N ha^{-1} was added at the time of seedling emergence. Spring barley (*Hordeum vulgare* L., cv. 'Nery') was grown as the non-fixing reference crop at the same N-levels as the pea crop. The experimental layout was nine randomized split-plot designs with crops as main plots and N-fertilizer levels as subplot and four replicates. Each subplot consisted of ten rows of length 4.40 m which were spaced 15 cm apart.

The crops were sown on 16 April with a ten-rowed Øyjord drill and seeding rates corresponded to 80 and 350 emerged pea and barley plants per m^2 , respectively. On 30 April 88 ± 5 (\pm SE) pea seedlings m^{-2} had emerged. Pea and barley seeds were treated with 'Thiram 80' and 'Baytan', respectively, before sowing.

The ^{15}N -labelled N-fertilizer was applied at rates corresponding to 25 or 50 kg N ha^{-1} two days after seedling emergence. The N-fertilizer consisted of a mixture of KNO_3 and $\text{Ca}(\text{NO}_3)_2$ with ^{15}N enrichments of 0.97 - 3.83 atom % ^{15}N excess. The labelled fertilizer was added to a microplot (6 rows of 1.2 m within the mainplot) as an aqueous solution distributed by a spray. Unlabelled $\text{Ca}(\text{NO}_3)_2$ was applied to the remaining part of the plot. The plots were sprayed with pesticides to control weeds and leaf eating weavills.

Sampling procedures

Nine harvests were taken during the growth season. From 40 cm of the central two rows in the ^{15}N -microplot plants were cut 2 cm above ground and collected for ^{15}N -analysis. Plants in the guard rows were discarded before the plants from the remaining area were collected and weighed. At the first four harvests only a combined above-ground biomass yield was determined. In harvest 5 to 8 plants were separated in vegetative organs and pods. At the last harvest, at physiological maturity in pea, pods were further separated in seeds and pod walls.

The leaf area was determined on plants from harvest 1 to 7. Three to five plants were randomly selected from each plot and the area of stipules and leaflets were measured by a Delta-T Device Areameter. The leaf area indexes (LAI) of green and yellow leaves were calculated assuming a plant stand of 88 plants m^{-2} .

Root length of pea was determined at the full bloom growth stage (56 days after seedling emergence). Four soil cores (diameter 62.5 mm) were taken within and between rows to a depth of 30 cm in each plot. The cores from each plot were mixed after division into 0-15 and 15-30 cm sections. Roots were washed with water to remove soil, and then collected and spread uniformly on a 500 μm sieve. Root length was determined according to Newmann (1966).

Dry matter and nitrogen determinations

Dry matter was determined after heating for 20 h at 80°C in a well-aerated oven. Total N was determined by a semi-micro Kjeldahl method including nitrate (Bremner and Mulvaney, 1982). ^{15}N -analysis was carried out on ammonium removed from the Kjeldahl digest by distillation and collection in 0.5 N H_2SO_4 . Aliquots corresponding to about 1 mg N were evaporated to dryness in ampules of Jena-glass and combusted with CuO and CaO at 560°C for 3 h in the evacuated and sealed ampule (modified Dumas) (Fiedler and Proksch, 1975). The $^{15}\text{N}:^{14}\text{N}$ ratio of the gas samples were determined by mass spectrometry. The atomic % ^{15}N excess was obtained by subtracting the natural abundance of ^{15}N (0.366 %) from the atomic % ^{15}N of the sample.

Calculations and statistical analysis

Nitrogen fixation was estimated according to the 'A'-value principle (Fried and Broeshart, 1975). Mean 'A'-values were calculated from the fertilizer-and soil-N uptake in spring barley supplied with 25 or 50 kg N ha^{-1} . Estimates of N_2 fixation were corrected for seed-borne N, assuming that 50% of the 9.6 kg seed-borne N ha^{-1} was located in the above-ground plant parts (Jensen et al., 1985). N_2 fixation in the pea crop receiving no labelled N-fertilizer was estimated under the assumption that the soil N uptake was equal to the mean soil N uptake by the fertilized peas. The crop growth rate, rate of N-assimilation, N_2 fixation rate and N-fertilizer uptake rate were calculated as:

$$\frac{q_2 - q_1}{t_2 - t_1} \quad (1)$$

where q_1 and q_2 are the quantities of above-ground dry matter or nitrogen in crops harvested t_1 and t_2 days after seedling emergence. Analysis of variance was carried out on the data.

Results

Growth of pea

The results of the growth analysis are presented in Fig. 1 as means of N-levels, because the effect of "starter" N was not statistically significant.

The growth curve of the field-grown pea had the typical sigmoid form (Fig. 1a). Maximum dry matter accumulation in above-ground plant parts (12.2 t ha^{-1}) was reached 90 days after seedling emergence (DAE), but the crop was still accumulating nitrogen when the final harvest was taken at physiological maturity (Fig. 1a). Total N accumulation at maturity was 314 kg N ha^{-1} , of which 265 kg N ha^{-1} was found in the seed. The mean seed yield was $6.7 \pm 0.4 \text{ t ha}^{-1}$.

The pea crop reached maximum leaf area index (LAI) 10 weeks after seedling emergence, but maximum green LAI of 6.8 one week earlier at the full bloom/flat pod growth stage (Fig. 1b). The crop growth rate (CGR) and rate of nitrogen assimilation (RNA) changed almost in parallel (Fig. 1c). CGR and RNA were slightly decreased when plants began to flower, but reached maxima of $480 \text{ kg DM ha}^{-1} \text{ day}^{-1}$ and $11.4 \text{ kg N ha}^{-1} \text{ day}^{-1}$, respectively, at the flat pod growth stage (Fig. 1c).

The onset of pod-filling (56-63 DAE) and the commencement of lodging reduced the rate of N-assimilation dramatically (Fig. 1c), and the mobilization of nitrogen from vegetative organs was initiated (Fig. 1e), whereas a decrease in dry matter of vegetative organs was not initiated until two weeks later (Fig. 1d). The pod harvest index of dry matter and N increased linearly until 90 days after emergence (Figs. 1d and 1e). At maturity harvest indexes of dry matter and nitrogen in seeds were 57 and 80%, respectively. Partitioning of dry matter and nitrogen, number of pods/ m^2 (760), number of seeds per pod or the mean seed weight (275 mg) were not significantly influenced by N-fertilization. The effect of N-fertilizer on pea root growth was studied on plants harvested 8 weeks after seedling emergence (Table 1). Measurement of root length and root biomass at this growth stage may give a rather good indication of total root growth, since it has been observed that pea root growth almost stops when plants begin to flower (Salter and Drew, 1965). "Starter" N tended to stimulate the root growth in the top 15 cm of the soil, but the effect was not significant (Table 1).

Fertilizer-N uptake

Only a few mm of rain fell during the first three weeks of growth (Fig. 3c). Since the main part of the ^{15}N -labelled N-fer-

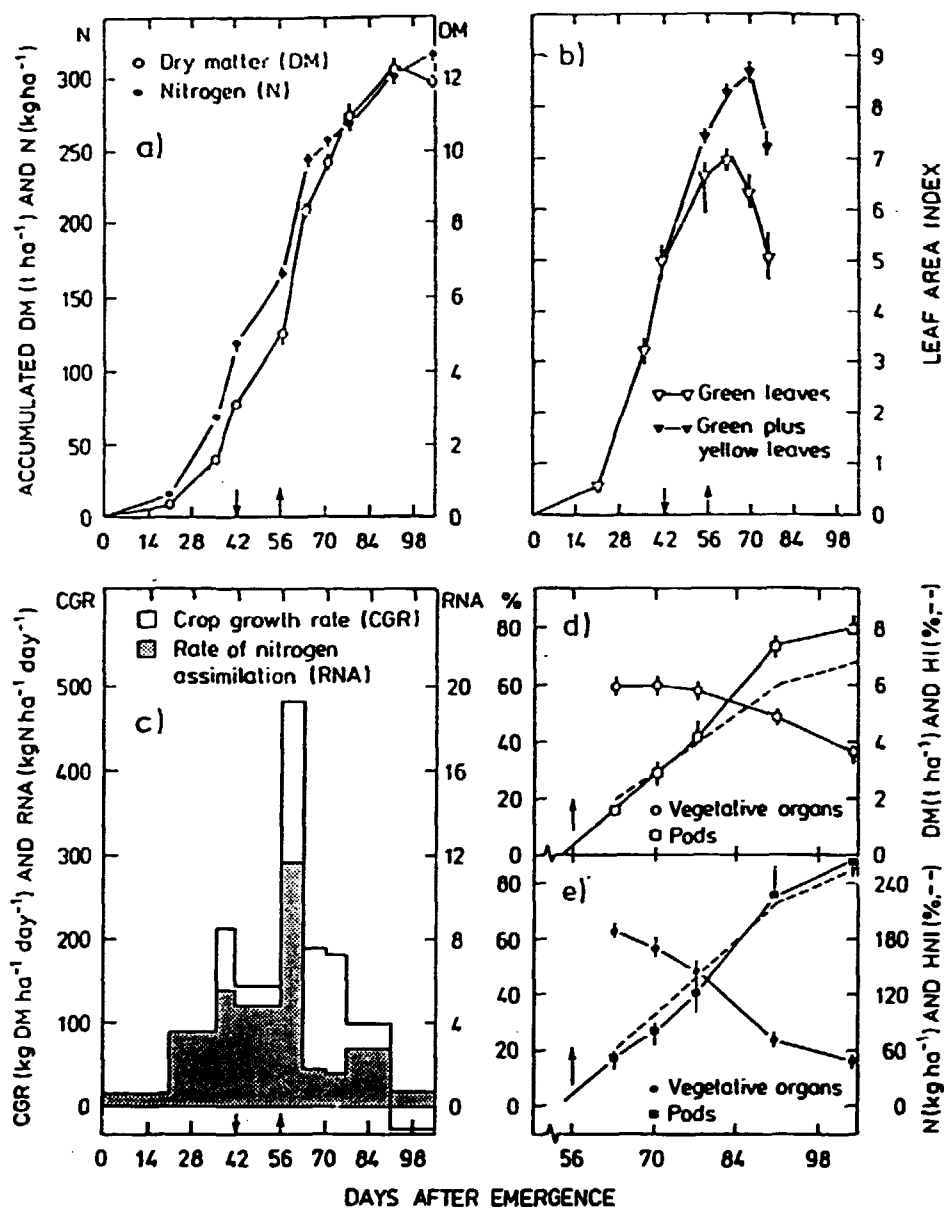


Fig. 1. Growth analysis of pea grown in the field; (a) accumulation of dry matter and nitrogen in above-ground plant parts, (b) leaf area index of green leaves and total leaf canopy, (c) crop growth rate (CGR) and rate of N assimilation (RNA), (d) partitioning of dry matter between pods and vegetative organs and the pod harvest index of DM, and (e) partitioning of nitrogen between pods and vegetative organs and the pod harvest index of nitrogen.

Data are means of N-fertilizer levels and replicates. Vertical bars indicates standard errors ($n=12$) and arrows the period of flowering.

Table 1. Root density, root biomass and root length of pea 56 days after seedling emergence as influenced by fertilizer N. Means \pm SE (n=4)

Fertilizer N kg ha ⁻¹	Soil depth cm	Root density cm cm ⁻³	Root biomass t DM ha ⁻¹	Root length* km m ⁻²
0	0-15	1.41 \pm 0.15	0.99 \pm 0.12	2.12
	15-30	0.72 \pm 0.11	0.37 \pm 0.05	1.08
25	0-15	1.55 \pm 0.09	1.06 \pm 0.10	2.33
	15-30	0.75 \pm 0.15	0.32 \pm 0.05	1.13
50	0-15	1.74 \pm 0.22	1.19 \pm 0.09	2.61
	15-30	0.56 \pm 0.05	0.41 \pm 0.07	0.84

* Calculated from the root density and root biomass measurements.

tilizer probably was located close to the soil surface, the fertilizer N-uptake was rather low during this period. Rainfall 21-28 DAE stimulated the fertilizer N-uptake (Figs. 2 and 3c). The patterns of fertilizer N-uptake in pea and barley were almost identical, but the recovery of fertilizer-N was higher in barley than in pea (Fig. 2). Maximum recovery of fertilizer-N in barley and pea was reached 56 DAE (Fig. 2). In pea the percentages recoveries of N after 25 and 50 kg applications were 76.0 ± 5.5 and 70.2 ± 2.8 , respectively (Fig. 2). From this growth stage until maturity there was a net loss of fertilizer-N from the above-ground plant parts in pea and barley, most pronounced in barley supplied with 50 kg N ha^{-1} (Fig. 2). The final percentage recoveries of fertilizer-N were 70.5 ± 5.2 and 68.2 ± 3.3 in pea and 63.6 ± 4.8 and 72.0 ± 1.9 in barley, for applications of 25 and 50 kg N ha^{-1} , respectively.

Symbiotic N_2 fixation

Plants with intact root systems were dug from the field three weeks after emergence to evaluate the effect of "starter" N on nodule formation. The N-fertilizer influenced neither the nodule mass ($21 \text{ mg dry weight plant}^{-1}$) nor the nodule number (33 per plant). However, symbiotic N_2 fixation was inhibited by application of "starter" N (Table 2). At the end of flowering (56 DAE) the average nitrate fertilizer inhibition of N_2 fixation was about 30%, thereafter the inhibition gradually decreased, being only 2 and 13% at maturity, for the 25 and 50 kg N ha^{-1} applications, respectively (Table 2).

About 25% of the total N_2 fixation took place during pre-flowering, about 25% during the two weeks of flowering and the remaining during post-flowering (Table 2).

Seasonal N_2 fixation rates were calculated from accumulated fixation (Fig. 3). In the unfertilized pea crop the N_2 fixation rate gradually increased to a maximum during early pod formation (56-63 DAE) when 10.3 kg N was fixed per ha per day (Fig. 3). This was also the time of maximum CGR and green LAI (Figs. 1b and 1c). At the initiation of lodging of the crop and early pod-filling (63-70 DAE) there was a steep decrease in the rate of N_2 fixation, but during later stages of pod-filling some N_2 fixing

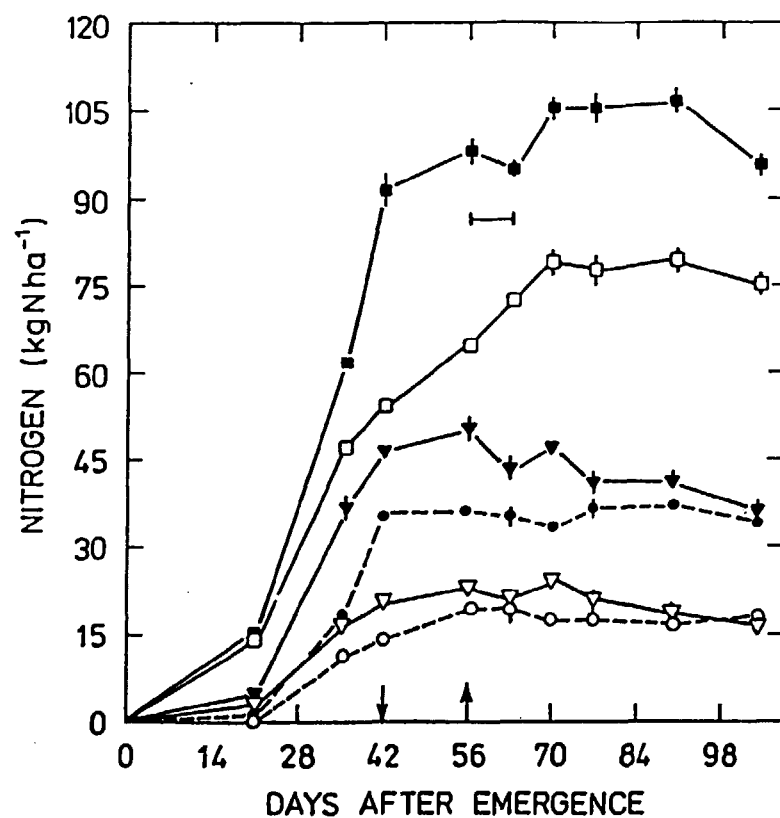


Fig. 2. Uptake of fertilizer-N in above-ground plant parts of pea (O, ●) and spring barley (nonfixing reference crop, ▽, ▼) and total N accumulation in above-ground barley plants parts (□, ■). O, ▽, □, 25 kg N ha⁻¹; ●, ▼, ■, 50 kg N ha⁻¹. Vertical bars indicates stand errors (n=4). Horizontal bar indicates anthesis in barley and arrows the period of flowering in pea.

Table 2. Estimated N_2 fixation in pea as influenced by addition of fertilizer-N.

Days after emergence	N supply (kg N ha ⁻¹)			LSD 0.05
	0*	25	50	
21	9	4	7	ns**
36	43	37	39	ns
42	65	53	42	ns
56	141	100	94	28
63	213	175	164	37
70	208	207	171	23
77	222	210	184	32
90	244	224	214	ns
104	244	238	213	18

* Calculated under the assumption that the soil N uptake is equal to the mean estimated soil N uptake in fertilized peas.

** not significant

activity was resumed (Fig. 3). The marked reduction of the N_2 fixing activity was simultaneous with a period of low rainfall (Fig. 3). The negative rate of N_2 fixation at 60-70 DAE may be due possibly to mobilization of nitrogen from above-ground plant parts for root growth.

The rate of N_2 fixation in pea was inhibited by the applied NO_3^- -N only during the period of maximum fertilizer N-uptake rates and the following few weeks (Fig. 3). Thereafter the N_2 fixing activity in N-fertilized pea resumed to approximately the same level as in pea receiving no N-fertilizer (Fig. 3).

The proportion of nitrogen derived from air (% Ndfa) was at maturity estimated to be 79, 74 and 68% of total crop nitrogen, respectively, in peas supplied with 0, 25 and 50 kg N ha⁻¹. However, nitrogen fixation did not contribute equally to total N in vegetative and reproductive plant parts (Table 3). There was thus a decline in the % Ndfa in the vegetative plant parts after 70 DAE, whereas the % Ndfa in pods were almost constant, being 79 and 73% in pea supplied with 25 and 50 kg N ha⁻¹, respectively (Table 3).

The accumulation of nitrogen from the different N-sources in the pea crop supplied with 25 kg N ha⁻¹ is shown in Fig. 4. Approximately 60 kg N ha⁻¹ was derived from the soil.

Discussion

The growth analysis confirmed previous observations in a vining pea (Milbourn and Hardwick, 1968) that maximum crop growth rate and maximum leaf area index are reached 2 to 3 weeks after flower initiation. The peak crop growth rate probably was associated with high photosynthetic rates in leaflets during early pod development (Flinn, 1974; Bethlenfalvay and Phillips, 1977). Thereafter, the weight of the pods caused lodging of the crop, probably with a concomitant reduction of photosynthesis and the crop growth rate.

The combination of frequent harvests and the use of the ¹⁵N isotope dilution technique made it possible to establish profiles of N_2 fixation rates during ontogeny (Fig. 3a). The seasonal profiles of N_2 fixation in field-grown pea were consistent with profiles obtained using the acetylene reduction assay on pot-grown

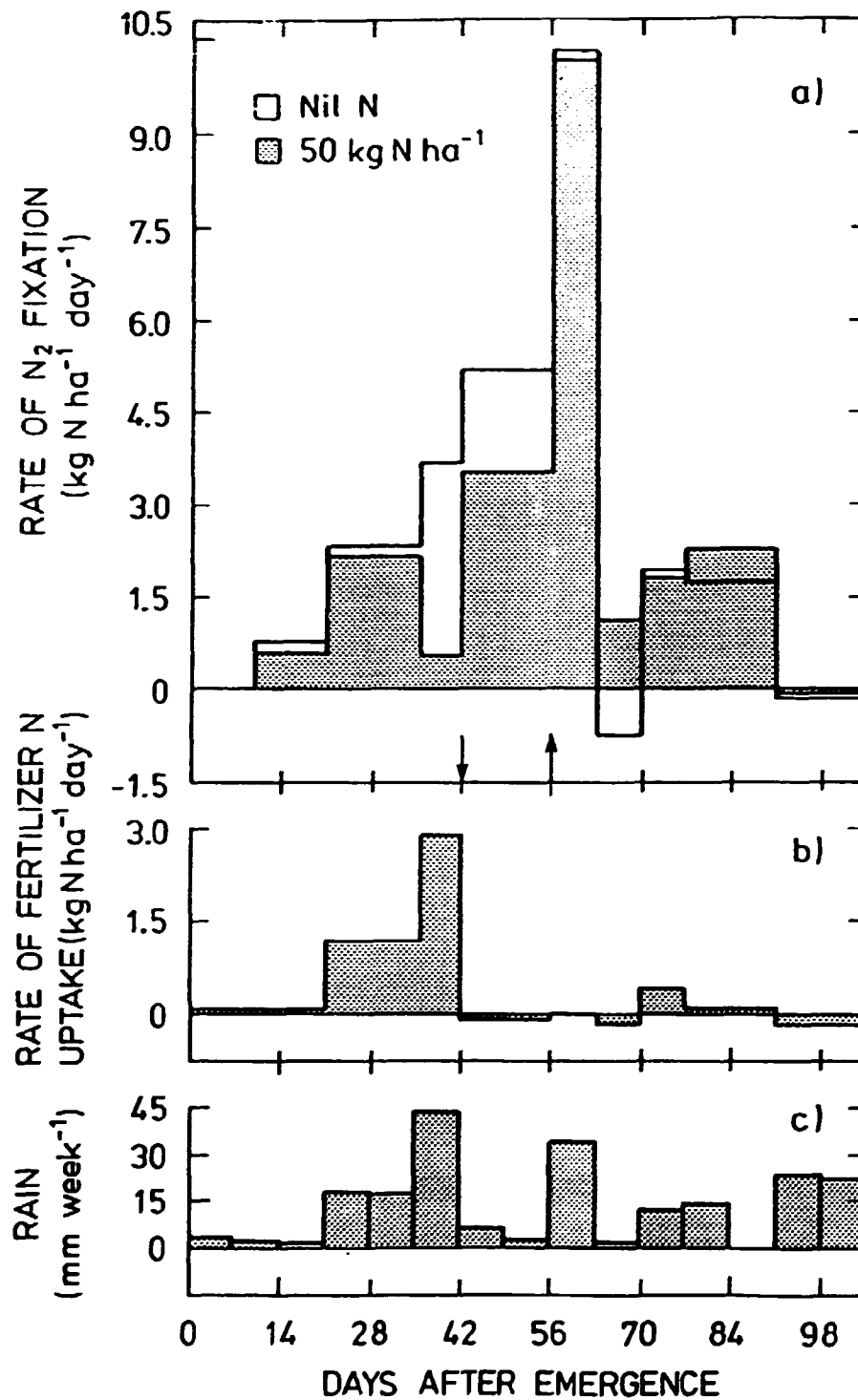


Fig. 3. Seasonal rates of N_2 fixation in pea without and with the supply of 50 kg $\text{NO}_3\text{-N ha}^{-1}$ (a); seasonal rates of fertilizer N (50 kg N ha^{-1}) uptake in pea, and (b); weekly accumulated rainfall (mm). Arrows indicates the period of flowering.

Table 3. Contribution from symbiotic N₂ fixation to total N in pea vegetative parts and pods (% of total N from N₂ fixation). Means \pm SE (n=4).

Days after emergence	N supply (kg N ha ⁻¹)			
	25		50	
	Vegetative parts	Pods	Vegetative parts	Pods
21	29 \pm 10	-	43 \pm 8	-
36	55 \pm 9	-	52 \pm 6	-
42	57 \pm 10	-	38 \pm 4	-
56	63 \pm 7	-	57 \pm 4	-
63	70 \pm 4	79 \pm 5	65 \pm 8	73 \pm 4
70	75 \pm 3	80 \pm 3	66 \pm 2	74 \pm 1
77	70 \pm 7	79 \pm 3	63 \pm 7	71 \pm 3
90	65 \pm 3	80 \pm 3	56 \pm 2	73 \pm 3
104	57 \pm 6	77 \pm 3	50 \pm 6	72 \pm 2

plants (e.g. Young, 1982), indicating peak N_2 fixation activity around flowering to early pod development. In the present experiment the rate of N_2 fixation was decreased simultaneous with lodging of the crop, with a reduction of the crop growth rate (Fig. 1c) and the leaf area (Fig. 1b), and with the initiation of mobilization of N from vegetative organs (Fig. 1e). These factors in combination may have caused a reduction in the amount of photosynthates translocated to nodules.

A high level of combined N usually inhibits N_2 fixation in legumes. The profiles of N_2 fixation (Fig. 3a) shows that there was an inhibition by nitrate during the period of growth, where the fertilizer-N was taken up, and a shortly after. This indicated that the symbiotic system quickly recovered from the effect of nitrate. The supply of 50 kg N ha^{-1} caused a 13% reduction of accumulated fixed N at maturity, which is consistent with previous studies on the effect of N-fertilization in field pea (Jensen, 1986a). Since total N-accumulation at maturity was not significantly influenced by N-fertilizer, the fertilizer-N only substituted for N_2 fixed symbiotically.

Previous studies on N_2 fixation in pea using barley as a non-fixing reference crop in the ^{15}N isotope dilution technique indicated that barley was not an ideal reference for pea (Witty, 1983; Jensen, 1986a). Figure 2 shows the patterns of fertilizer-N uptake in pea and barley. The crops started the uptake of fertilizer-N simultaneously, and maximum N-fertilizer content in the crops were reached at about the same time (Fig. 2). This indicates that the patterns of N-uptake from the soil N pool in the two crops may not be very different. Studies on the rooting patterns of barley and pea have indicated that barley on clay soil has a greater rooting depth than pea, but on sand soils the two crops have similar rooting depths (Vetter and Scharafat, 1964). Since the present experiment was carried out on a loamy sand soil the two crops may have been able to take up soil N from an equal soil volume. This indicates that barley may not be as poor a reference for pea as has previously been indicated (Witty, 1983; Jensen, 1986a).

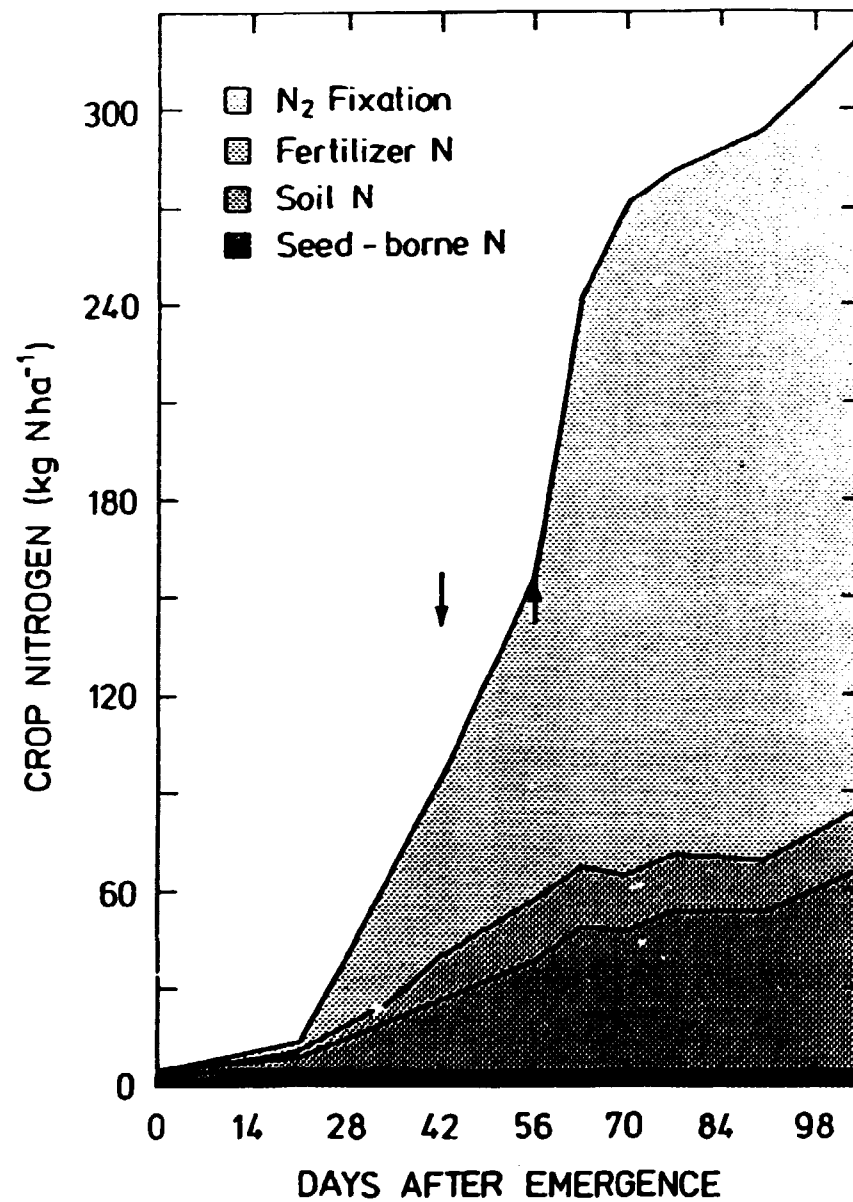


Fig. 4. Origin of N in the above-ground biomass of pea (fertilizer N: 25 kg N ha⁻¹). Arrows indicate the period of flowering.

The partitioning of N and dry matter are important processes in determining the dry matter and protein seed yield of dry peas and other legumes. The transport of nitrogen from vegetative organs was initiated before the redistribution of photosynthetic assimilates fixed during vegetative growth (Fig. 1d and 1e). Furthermore, the rate of nitrogen allocation to pods seemed to be faster than the rate of dry matter allocation to pods. This was deduced from the slopes of the pod harvest index curves during the linear increase (Spaeth and Sinclair, 1985) (Fig. 1d and 1e).

Assuming that the amount of nitrogen that is mobilized from vegetative organs to pods is identical to the decline in the nitrogen content of vegetative organs from pod initiation to maturity, it was estimated that about 55% of total pod N should be derived from above-ground vegetative organs (Table 4). Almost all of the fertilizer-N in pods were derived from vegetative organs (Table 4). Since no account was taken of nitrogen mobilized from the root system, the estimate of that 45% of total pod N should be derived from assimilation of N during the period from pod initiation to maturity, may actually be too high. Thus, the results are in agreement with data reported by Pate (1985), concluding that about 35% of total pod N in a determinate pea cultivar was derived from N assimilated post-flowering and 13% is mobilized from the roots.

The supply of "starter" N increased the early dry matter production and the leaf area slightly, but these observations were not statistically significant. That fertilizer-N applied at sowing had no significant effect is in agreement with previous studies on N-fertilization of pea (Andersen *et al.*, 1983; Jensen 1986a; 1986b), and with Pate (1958), who observed that nodules were developed before the exhaustion of cotyledons for N. Nitrogen starvation during early growth in pea, as observed by Mahon and Child (1979), may take place only under extreme conditions where no combined N is available immediately after germination.

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Symbiotic N_2 fixation in pea and field bean estimated by ^{15}N fertilizer dilution in field experiments with barley as a reference crop

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Key words A-value Barley Field bean Isotope dilution Nitrogen fixation ^{15}N
Non-fixing reference crop Pea *Pisum sativum* Vicia faba

Summary The total amount of nitrogen derived from symbiotic nitrogen fixation in two pea and one field bean cultivar, supplied with 50 kg N ha^{-1} at sowing ('starter'-N), was estimated to 165, 136, and 186 kg N ha^{-1} , respectively (three-year means). However, estimates varied considerably between the three years. At the full bloom/flat pod growth stage from 30 to 59 per cent of total N_2 fixation had taken place. The proportion of total N derived from N_2 fixation at maturity was higher in seeds than in vegetative plant parts and amounted to 59.5, 51.3 and 66.3 per cent of total above-ground plant N in the two pea cultivars and field bean, respectively (three-year means). The recovery of fertilizer N was 62.2, 70.2, 52.1, and 69.5 per cent in the two pea cultivars, field bean and barley, respectively. Growth analysis indicated that barley did not meet the claims for an ideal reference crop in the ^{15}N fertilizer dilution technique for estimating N_2 fixation in pea and field bean. 'Starter'-N neither increased the seed yield nor the N content of the grain legumes.

Introduction

Pea and field bean obtain their nitrogen from symbiotic N_2 fixation in root nodules formed by *Rhizobium leguminosarum* and from nitrate and ammonium originating from mineralization of soil organic matter and occasionally also from applied N-fertilizer depending on the farming praxis.

The published estimates of symbiotic N_2 fixation in pea are very inconsistent. Estimates based on the acetylene reduction assay⁸ indicated that only 33% of total N was derived from fixation. However this assay is not suitable for estimating integrated values of seasonal N_2 fixation¹⁶. Lysimeter-studies¹³ with $^{15}N_2$ indicated on the contrary that pea derived 93% of their total N from fixation, even when the soil contained considerable amounts of combined N. Using the ^{15}N fertilizer dilution technique Witty¹⁶ found that 58% of total N in a pea crop supplied with 30 kg N ha^{-1} was derived from fixation. In field beans the proportion of total plant N deriving from nitrogen fixation is found to 60–70% in several reports^{3, 4, 12, 16}.

The purpose of this study was to evaluate the contributions from

the different N-sources: soil-N, fertilizer-N and N_2 fixation to the nitrogen supply of two field pea cultivars with different growth habits and an early field bean cultivar. The effect of a small initial N-fertilizer addition ('starter'-N) on the yield of these grain legumes was also investigated. The ^{15}N fertilizer dilution technique was used with barley as non-fixing reference crop.

Methods

Site

The experiments were carried out during the years 1980, 1981 and 1982 in the Risø experimental fields. The soil was a sandy loam with pH about 7. Before sowing the soil was sampled for an analysis of extractable $NO_3-N + NH_4-N$ (soil:2 N KCl = 1:10) and 30 kg P and 50 kg K ha^{-1} was applied in PK-fertilizer. The soil contained 100, 54 and 98 kg $NO_3-N + NH_4-N ha^{-1}$ in the upper 40 cm of the profile in 1980, 1981 and 1982, respectively.

Crop plants

Nitrogen fixation was studied in two field pea (*Pisum sativum* L.) cultivars, 'Bodil' and 'Timo' and in the field bean (*Vicia faba* L.) cultivar 'Diana'. 'Bodil' is an early dwarf pea cultivar with white flowers and 'Timo' is a tall pea cultivar with purple-violet flowers. 'Diana' is a relatively early and small-seeded field bean cultivar. Spring barley (*Hordeum vulgare* L. cv. 'Nery') was grown as non-fixing reference crop.

Experimental design

The crops were grown at two levels of N-fertilizer addition, no (N_0) and 50 kg $N ha^{-1}$ (N_{50}) in a randomized complete split-plot design with six replicates. In 1980 additional nine replicates were included for growth analysis. Treatments were arranged with N-fertilizer levels as main plots and grain legumes/barley as subplots. Each subplot consisted of ten rows of length 3.2 m, spaced 14.3 cm apart.

Plot preparation

Peas, field bean and barley were sown with a ten-rowed Øyjord drill. Seeding rates corresponded to approximately 80, 60 (40 in 1981) and 350 emerged plants m^{-2} of peas, field bean and barley, respectively. Dates of sowing and emergence are shown in Table 1. Two weeks after sowing ^{15}N -labelled N-fertilizer was applied at a rate corresponding to 50 kg $N ha^{-1}$. The ^{15}N -labelled fertilizer (a mixture of KNO_3 and $Ca(NO_3)_2$ with an ^{15}N atom % excess of about 3.8) was added to a microplot (6 rows of 1.2 m within the main plot) as an aqueous solution distributed by a spray. Unenriched $Ca(NO_3)_2$ was applied to the remaining part of the plot and the nitrogen fertilizer was immediately watered in with 10 l of tap-water.

Harvests and nitrogen determination

Crops were harvested at the full bloom/flat pod growth stage (Harvest 1) and at physical maturity (Harvest 2) to determine the relationship between growth stage and N_2 fixation. At maturity field bean leaves were not included. See Table 1 for dates of harvests. In 1980 three additional harvests were taken, one before and two after the full bloom/flat pod growth stage. At each harvest three replicate plots were harvested by hand. Plants from 40 cm of the central two rows of the ^{15}N -microplot were removed for ^{15}N -analysis. Plants from the guard rows were discarded before the plants from the remaining 2.1 m^2 were collected and weighed. At the first harvest (and the 3 additional harvests in 1980) only the yield of shoots was determined. At the harvest at maturity crops were separated in seeds, hulls and stem + leaves. After dry matter determination (80°C for 20 h in a well-aerated oven) the samples were ground and total N determined by a semi-micro Kjeldahl procedure including nitrate¹. ^{15}N -analysis was

Table 1. Dates of sowing, seedling emergence and harvest in the three experimental years

Year and crop	Sowing	Seedling emergence	Harvest	
			Full bloom*	Maturity
1980				
Bodil pea	16/4	6/5	24/6	8/8
Timo pea	16/4	6/5	1/7	8/8
Field bean	16/4	6/5	1/7	27/8
Barley	16/4	6/5	24/6	8/8
1981				
Bodil pea	13/4	7/5	24/6	17/8
Timo pea	13/4	7/5	1/7	17/8
Field bean	13/4	7/5	1/7	3/9
Barley	13/4	7/5	24/6	17/8
1982				
Bodil pea	7/4	25/4	24/6	4/8
Timo pea	7/4	25/4	1/7	11/8
Field bean	7/4	25/4	1/7	11/8
Barley	7/4	25/4	24/6	4/8

*The full bloom/flat pod growth stage in legumes and anthesis in barley.

carried out on ammonium originating from plant samples harvested on the microplots. Ammonia was removed from the Kjeldahl digests by distillation and collected in 0.5 *N* H₂SO₄. Aliquots hereof corresponding to about 1 mg N were evaporated to dryness in ampoules of Jena glass and combusted with CuO and CaO at 560°C for 3 h in the evacuated and sealed ampoule (modified Dumas)². The ¹⁵N:¹⁴N ratios of the gas samples were determined by mass spectrometry. The atom % ¹⁵N excess was obtained by subtracting the natural abundance of ¹⁵N (0.366%) from the atom % ¹⁵N of the sample.

Calculations

Nitrogen in the legumes derived from fertilizer, soil and symbiotic nitrogen fixation was estimated using the 'A'-value concept^{1, 4} and barley as the non-fixing reference. Estimates were corrected for seed-borne nitrogen. A seed of pea, field bean and barley, respectively, contained normally 10, 15 and 1 mg N. It was found⁵ that the seed-borne nitrogen became distributed evenly between above- and below-ground plant parts. Estimates of the contributions from the different N sources to total N were not treated statistically because of different harvest times of the crops and several assumptions underlies the estimates.

Results and discussion

Crop yield and N uptake

An initial supply of 50 kg N ha⁻¹ ('starter'-N) did not significantly influence the seed yields, which therefore are shown as means of N-levels in Table 2. Levels of combined soil N, derived from mineralization of organic matter, were probably high enough for supporting nitrogen nutrition of the grain legumes until nodules were formed and thus overcome N-stress, which may occur at low combined N-levels⁹, during early development. In other investigations^{10, 12} it was also reported that pea and field bean did not respond to N-fertilizer applied at sowing, when conditions for symbiotic nitrogen fixation were favorable.

Patterns of nitrogen uptake in the grain legumes and the reference crop during the 1980 growth season are shown in Fig. 1. In field bean the decline in total N content after 105 days from emergence was associated with loss of dead leaves. In 1982 fallen field bean leaves sampled at maturity contained 33 kg N ha^{-1} .

Table 2. Yield of seed dry matter (t ha^{-1}) of grain legumes (means of N_0 and N_{50}) and barley (N_{50})

Crop	Year		
	1980	1981	1982
Bodil pea	4.1	4.3	7.4
Timo pea	2.5	4.1	4.8
Field bean	5.3	5.3	4.7
LSD (0.05)	0.7	ns*	0.7
Barley	5.5	4.7	6.2

*ns: not significant.

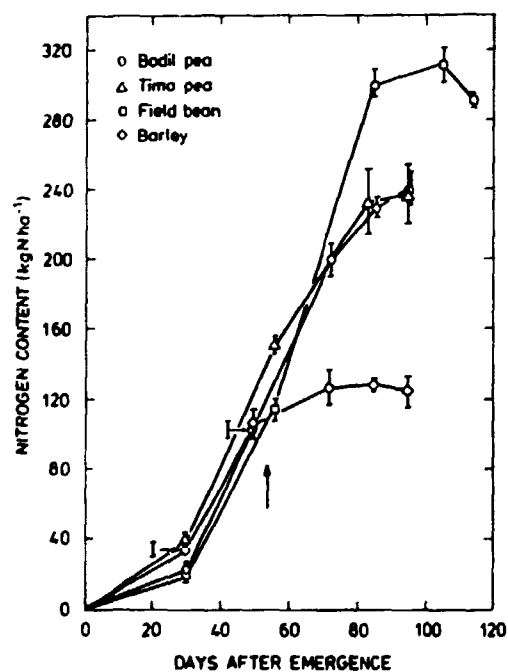


Fig. 1. Patterns of nitrogen uptake (N content of above-ground biomass) in grain legumes and barley during the 1980 growth season. Crops were supplied with 50 kg N ha^{-1} at sowing. Bars indicate S.E. of means ($n = 3$); arrow indicates harvest at the full bloom/flat pod growth stage in legumes and anthesis in barley.

In 1980 and 1981 the highest N content at maturity was found in field bean (Table 3). This was probably due to the longer period of growth compared to pea. Since symbiotic nitrogen fixation in field bean is very sensitive to water stress¹⁴, dry conditions during the end of July and early August in 1982, probably reduced the symbiotic nitrogen fixation, and resulted in a very early maturation of the field bean plants. On the contrary the climatic conditions in 1982 were excellent for the growth of the pea crops, which accumulated more nitrogen (Table 3) and yielded more (Table 2) this year than in 1980 and 1981.

The nitrogen content of the above-ground biomass at both harvests (Table 3) and the N-concentration of the seeds (data not shown) were not significantly influenced by fertilizer nitrogen supplied at sowing.

Table 3. Nitrogen content (kg N ha⁻¹) of above-ground biomass of crops at the two harvest times

Crop	N-fertilizer kg N ha ⁻¹	Year		
		1980	1981	1982
<i>Full bloom/flat pod stage</i>				
Bodil pea	0	91	120	145
	50	104	102	151
Timo pea	0	142	148	181
	50	149	134	206
Field bean	0	106	67	139
	50	116	55	150
LSD (0.05)		18	19	16
<i>Maturity</i>				
Bodil pea	0	259	240	323
	50	242	244	337
Timo pea	0	224	273	284
	50	235	226	323
Field bean*	0	303	294	267
	50	293	299	252
LSD (0.05)		42	65	49

* Fallen leaves not included.

Sources of nitrogen

The atom % ¹⁵N excess of legume plant parts were lower than of barley plant parts (Table 4), as a result of the symbiotic nitrogen fixation in the legumes. The atom % ¹⁵N excess was reduced from the full bloom/flat pod growth stage to maturity (Table 4). At maturity the atom % ¹⁵N excess in different legume plant parts were non-uniform (Table 4), and could be ranked in the following order: stem + leaves > hulls > seeds.

The atom % ¹⁵N excess of barley plant parts at maturity was also

Table 4. Atom per cent ^{15}N excess in different plant parts (means \pm standard deviation)

Year and crop	Harvest I [*]	Maturity		
	Shoot	Stem + leaves ^{**}	Hulls ^{***}	Seeds
1980				
Bodil pea	0.98 \pm 0.07	0.68 \pm 0.08	—	0.49 \pm 0.03 ^{****}
Timo pea	0.86 \pm 0.04	0.68 \pm 0.03	—	0.63 \pm 0.03
Field bean	0.77 \pm 0.16	0.43 \pm 0.01	—	0.34 \pm 0.01
1981				
Bodil pea	0.71 \pm 0.12	0.50 \pm 0.09	0.47 \pm 0.10	0.43 \pm 0.13
Timo pea	0.62 \pm 0.08	0.64 \pm 0.09	0.49 \pm 0.13	0.48 \pm 0.13
Field bean	0.57 \pm 0.07	0.44 \pm 0.03	0.37 \pm 0.06	0.28 \pm 0.05
1982				
Bodil pea	0.72 \pm 0.09	0.56 \pm 0.06	0.37 \pm 0.05	0.34 \pm 0.04
Timo pea	0.65 \pm 0.07	0.54 \pm 0.01	0.37 \pm 0.03	0.35 \pm 0.04
Field bean	0.61 \pm 0.03	0.51 \pm 0.04	0.60 \pm 0.15	0.37 \pm 0.00
Barley				
1980	1.57 \pm 0.36	1.20 \pm 0.06	1.20 \pm 0.08	1.23 \pm 0.12
1981	1.33 \pm 0.09	1.07 \pm 0.08	1.01 \pm 0.09	1.07 \pm 0.16
1982	1.45 \pm 0.12	1.09 \pm 0.10	0.90 \pm 0.08	1.01 \pm 0.08

^{*}Full bloom/flat pod growth stage in legumes; anthesis in barley.

^{**}Field bean leaves not included.

^{***}Glume in barley.

^{****} ^{15}N -analysis was carried out on pods with seeds.

non-uniform and was reduced from anthesis to maturity (Table 4). This reflected the declining ^{15}N -enrichment of the N taken up with time, probably caused by mineralization of organic bound soil N.

Nitrogen fixation was estimated using the 'A'-value concept^{3,4}, in which the amount of plant available soil nitrogen ('A'-value) is calculated from the fertilizer and soil nitrogen uptake in a non-fixing reference crop. The soil N uptake in the legume is estimated from the recovery of N fertilizer in the legume and the calculated 'A'-value. Estimates of N_2 fixation are then obtained by subtracting nitrogen derived from fertilizer, soil and seed-borne nitrogen from total above-ground plant nitrogen. The principle described by Fried and Middelboe⁵, in which the proportion of total plant N derived from fixation (% Ndfa) is calculated as:

$$\% \text{Ndfa} = \left[1 - \frac{\text{atom } \% ^{15}\text{N excess (legume)}}{\text{atom } \% ^{15}\text{N excess (reference)}} \right] \times 100$$

was not used because the non-uniformity of atom % ^{15}N excess in barley plant parts could be expected to cause an error¹¹. Estimating N_2 -fixation according to this principle⁵ would further have required

that the atom % ¹⁵N excess of barley and legume plant parts were corrected for seed-borne nitrogen before calculations. This is because seed-borne N in different species dilute the ¹⁵N-enrichment to different extent. The use of the 'A'-value concept made it easy to correct the estimates of nitrogen fixation for seed-borne nitrogen. The amount of nitrogen taken up from fertilizer and soil by the reference crop and calculated 'A'-values are shown in Table 5. 'A'-value were increased from anthesis to maturity, probably due to mineralization of organic bound soil nitrogen.

Table 5. 'A'-values and nitrogen derived from various sources in above-ground biomass of barley

Year	Nitrogen derived from		Percent of fertilizer recovered*	'A'-values** (kg N ha ⁻¹)
	Soil (kg N ha ⁻¹)	Fertilizer (kg N ha ⁻¹)		
<i>Anthesis</i>				
1980	60 ± 20***	41 ± 3	85.6	74
1981	54 ± 3	29 ± 2	65.6	92
1982	52 ± 4	35 ± 5	76.1	75
<i>Maturity</i>				
1980	85 ± 12	40 ± 2	83.2	110
1981	69 ± 8	27 ± 2	57.6	126
1982	85 ± 15	32 ± 3	67.8	129

*¹⁵N recovered in above-ground biomass.

**'A'-values are calculated from data including nitrogen in roots and stubble.

***Means of three replicates ± standard deviation.

The estimates of the size of symbiotic N₂ fixation varied considerably between the three years, especially in the peas (Table 6). This was due to the varying climatic conditions during the growth periods and different contents of nitrate in the soil. It must be borne in mind, that the length of time from emergence to first harvest at the full bloom/flat pod growth stage varied during the three years (Table 1). Since this growth stage is associated with maximum nitrogenase activity^{7, 14} a few weeks difference in the length of this period, will have a significant effect on the estimates of N₂ fixation. Nitrogen fixation at the full bloom/flat pod growth stage represented 30, 59 and 31% of total nitrogen fixation at maturity in 'Bodil' pea, 'Timo' pea and field bean, respectively (three-year means)(Table 6).

'Timo' pea had taken up more fertilizer nitrogen than 'Bodil' pea at both harvest times (Table 6). The recovery of fertilizer N was 62.2 and 70.3% as three-year means, in 'Bodil' and 'Timo', respectively. The corresponding figure for field bean was only 52.1%. However in 1982 7.1% of the fertilizer N was recovered in fallen leaves, so there may not

Table 6. Proportions of nitrogen in legumes derived from various N-sources and the percentage of fertilizer N recovered at the two harvest times

Year and crop	Percent of fertilizer recovered	Percent of N derived from			N ₂ -fixation kg N ha ⁻¹
		fertilizer	soil	fixation	
<i>Full bloom/flat pod growth stage</i>					
<i>1980</i>					
Bodil pea	52.7	25.5	40.1	31.3	32
Timo pea	66.8	22.4	35.2	40.1	60
Field bean	46.6	20.0	31.6	44.1	51
<i>1981</i>					
Bodil pea	37.9	18.6	33.7	44.5	46
Timo pea	44.1	16.4	29.7	51.6	69
Field bean	16.4	15.0	27.0	52.6	29
<i>1982</i>					
Bodil pea	58.1	19.3	29.5	48.1	73
Timo pea	72.6	17.4	26.5	54.0	112
Field bean	49.1	16.4	25.0	55.7	83
<i>Maturity</i>					
<i>1980</i>					
Bodil pea	69.3	14.3	31.6	52.8	128
Timo pea	79.4	17.1	37.7	43.8	102
Field bean	54.0	9.2	20.3	68.8	202
<i>1981</i>					
Bodil pea	51.4	10.4	26.2	62.1	150
Timo pea	62.7	13.9	35.0	49.7	113
Field bean	48.2	8.1	20.5	70.2	211
<i>1982</i>					
Bodil pea	65.9	9.8	25.2	63.7	215
Timo pea	68.7	10.7	27.5	60.4	195
Field bean	54.0	10.7	27.7	59.8	150

have been any difference between 'Bodil' pea and field bean. These results are in contrast to Witty¹⁶, who found that the recovery of fertilizer N in field bean was twice that in peas.

The proportion of above-ground biomass N derived from nitrogen fixation (% Ndfa) at maturity was higher in 'Bodil' than in 'Timo' pea, 59.5 and 51.3%, respectively (three-year means). These results indicated that the pea cultivars differed in their ability to fix nitrogen symbiotically as well as utilize the combined N-sources available (Table 6). 'Timo', the tall indeterminate pea cultivar took up a higher proportion of the fertilizer N than 'Bodil' pea, the dwarf determinate cultivar. However nitrogen fixation and total N content of 'Bodil' pea was higher than of 'Timo' pea. In field bean the average % Ndfa was estimated to be 66.3%. The estimates of % Ndfa in peas and field bean reported here are in good agreement with other studies^{3, 15, 16} of nitrogen fixation by the ¹⁵N fertilizer dilution technique.

The % Ndfa in reproductive plant parts were higher than in vegetative plant parts; this was also indicated by the ¹⁵N percentages of those plant parts (Table 4). In seeds 62, 55 and 69% of total N was derived from nitrogen fixation, whereas 44, 42 and 56% of total stem + leaf N (three-year means) were derived from symbiotic nitrogen fixation in 'Bodil', 'Timo' and field bean, respectively.

Absolute values of nitrogen fixation in 'Bodil' pea, 'Timo' pea and field bean at maturity were estimated to be 165, 136 and 186 kg N ha⁻¹, respectively (three-year means) (Table 6). These values are higher than the majority of the values published previously^{3, 15, 16}.

Estimates of nitrogen fixation without the use of ¹⁵N-labelled fertilizer were obtained assuming that the soil N uptake in legumes with N-fertilizer was similar to that without. The mean values for three years of the nitrogen fixation in 'Bodil', 'Timo' and field bean in this way were found to be 195, 171 and 220 kg N ha⁻¹ fixed per growth season, which corresponded to 71, 66 and 70%, respectively, of total crop nitrogen. This indicates that N-fertilization with 50 kg N ha⁻¹ at sowing reduced the nitrogen fixation with 15, 20 and 15% in 'Bodil' pea, 'Timo' pea and field bean, respectively.

Barley as non-fixing reference crop

The pattern of N uptake in barley was very different from that of the legumes (Fig. 1 and Tables 3 and 5). At maturity in barley 81% of total N in above-ground biomass (three-year mean) were taken up at anthesis (Table 5). Maximum N-content in 1980 was reached 2–3 weeks before the maximum N content of the legumes (Fig. 1). This showed that barley took up the bulk of its nitrogen during the early part of the growth season, whereas only 44, 62 and 39% of total N at maturity was assimilated at the first harvest in 'Bodil', 'Timo' and field bean, respectively. Since it has been found¹⁶, that the ¹⁵N-enrichment of the inorganic soil N pool is declining during growth due to mineralization of organic bound nitrogen, a crop like barley with an early N uptake will take up nitrogen during a period with a high ¹⁵N-enrichment of the soil N pool. The grain legumes, which assimilate nitrogen more evenly over the whole growth season (Fig. 1 and Table 6) would consequently also take up nitrogen during the period with a lower ¹⁵N-enrichment of the soil N pool. 'A'-values obtained from the N uptake of barley may therefore be too low when used with the legume crops and consequently may lead to an overestimate of nitrogen fixation.

The amount of fertilizer N in barley was lower at maturity than at anthesis (Table 5). This may be due to loss of nitrogen in fallen leaves or leaching of nitrogen from dead leaves on the plant. The soil N uptake

(Ndfs) in barley was increased from anthesis to maturity. The amount of fertilizer N taken up by the grain legumes from the first harvest to harvest at maturity was contrary to barley increased (Table 6). Estimating 'A'-values from the N uptake in barley may have resulted in 'A'-values that were too high when used with the legumes; this consequently lead to an underestimate of nitrogen fixation. The rooting depth of barley is presumably greater than that of pea and field bean. Barley therefore may have taken up soil N from a greater N pool than the legumes and consequently the 'A'-values would have been too high when used with the legumes, and therefore also lead to an underestimate of nitrogen fixation. The different harvest times of the reference crop and 'Timo' pea and field bean may have resulted in an overestimation of N_2 fixation. To some extent these sources of error may have counter-balanced each other.

It is obvious from the above, that barley does not meet the claims stated by Witty¹⁶ for an ideal reference crop. The ^{15}N fertilizer dilution technique might be improved, however, by selecting a reference crop with a pattern of N uptake more similar to the legume in question.

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III

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The influence of rate and time of nitrate supply on nitrogen fixation and yield in pea (Pisum sativum L.).

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Key words: ^{15}N fertilizer dilution, nitrate-N, nitrogen fixation, pea, yield

Abstract. The influence of nitrate N supply on dry matter production, N content and symbiotic nitrogen fixation in soil-grown pea (Pisum sativum L.) was studied in a pot experiment by means of ^{15}N fertilizer dilution. In pea receiving no fertilizer N symbiotic nitrogen fixation, soil and seed-borne N contributed with 82, 13 and 5% of total plant N, respectively. The supply of low rates of nitrate fertilizer at sowing ("starter N") increased the vegetative dry matter production, but not the seed yield significantly. Nitrogen fixation was not significantly decreased by the lower rates of nitrate, but higher rates supplied at sowing reduced the nitrogen fixation considerably. Applying nitrate N at the flat pod growth stage increased the yield of seed dry matter and N about 30% compared to pea receiving no nitrate fertilizer. Symbiotic nitrogen fixation was reduced only about 11%, compared with unfertilized pea, by the lowest rate of nitrate at this application time. The pea very efficiently took up and assimilated the nitrate N supplied. The average fertilizer N recovery was 82%. The later the N was supplied the more efficiently it was recovered. When nitrate was supplied at the flat pod growth stage 88% was recovered, and 90% of this N was located in the seeds.

Introduction

Field pea (Pisum sativum L.) derives its nitrogen from the seed N store, soil N (NO_3 , NH_4) and symbiotic nitrogen fixation. In the period from exhaustion of the seed N store until nodules are formed and the symbiosis supplies the plant with reduced N, growth is dependent on soil NH_4 and NO_3 -N. A low level of combined N may stimulate the nodulation [13, 14] and the early leaf area development [11]. This stimulation may result in a greater capacity for symbiotic N_2 fixation during later growth stages. Therefore combined soil N derived from mineralization of organic N is sometimes supplemented with small doses of fertilizer N ("starter N") in order to secure a good establishment of the pea crop. However, when efficient populations of Rhizobium leguminosarum are present in the soil, pea rarely responds to N fertilization at sowing [14]. Higher levels of combined N reduces nodule formation and growth and the activity of nitrogenase [8, 12, 13].

Under controlled growth conditions it is often found that the dry matter production and total N accumulation of peas grown on combined N sources are higher than of symbiotically grown plants [11, 13]. This might be due to early N-stress in the symbiotically grown plants [11] or to higher carbohydrate costs of N_2 assimilation than of uptake and assimilation of nitrate [10].

At the end of flowering in pea the rate of symbiotic nitrogen fixation is decreased [9, E. S. Jensen unpublished] due to the accumulation of photosynthates in the centers where reproductive organs are developed [9]. Consequently nitrogen and other nutrients are translocated from vegetative plant parts to meet the nutrient demands of the reproductive organs. The remobilization of nutrients from the vegetative plant parts leads to reduced rates of photosynthesis and senescence of leaves. Supplementing N_2 fixation with foliar fertilization during reproductive development may extend the longevity of the photosynthetic system, the

seed-filling period and the yield of legumes [6]. However foliar N-fertilization may also depress yields due to leaf burning [19]. Some reports indicate that increasing the supply of combined N in the soil during reproductive development may increase the seed yield and N content [1, 17].

The objectives of this study were to determine the influence of rate and time of nitrate supply on dry matter production, N accumulation, nitrogen fixation and fertilizer N utilization in pea. The ^{15}N isotope dilution technique was used.

Materials and methods

The pot experiment was conducted in the open in cylindrical PVC-pots with a surface area of 500 cm^2 and a volume of 20 l. The growth medium was a 1/1 (w/w) mixture of a soil (with 16% clay) and coarse sand (22.5 kg/pot) with pH 7.5. The growth medium contained 2.1 mg NO_3 and NH_4 -N per 100 g dry weight (extractable with 2 M KCl). The growth medium received basic dressings of all micro- and macro nutrients except N.

The experiment consisted of nine fertilizer N treatments in a randomized block design with four replicates. The treatments were: a) NO_3 -N supplied at sowing ("starter N") in five rates (0, 0.3, 0.6, 1.2, and 2.4 g N/pot), b) 1.2 g NO_3 -N/pot supplied at the time of first bloom (47 days after seedling emergence), c) 1.2 and 2.4 g NO_3 -N/pot supplied at the flat pod growth stage (58 days after seedling emergence) and d) NO_3 -N supplied as three split applications of 0.8 g NO_3 -N/pot each, at sowing, at the time of first bloom and at the flat pod growth stage, respectively. The fertilizer N was supplied as a mixture of KNO_3 and $\text{Ca}(\text{NO}_3)_2$ with 7.80 atom % ^{15}N . N supplied at sowing was placed 15 cm below the soil surface; later applications were distributed at the soil surface. In the treatment with split supply, three subtreatments, which differed only with respect to the time the fertilizer added was labelled with ^{15}N , were included.

Seeds of a dwarf, determinate pea (Pisum sativum L., cv. 'Bodil') were sown and thinned to 14 plants per pot after seedling emergence. The seeds were inoculated with Rhizobium leguminosarum strain Risø 1a. Pots were watered to a pre-determined weight two to three times a week. Lodging was prevented by wire frames.

Spring barley (Hordeum vulgare L., cv. 'Nery') supplied with 2.4 g NO₃-N/pot at sowing was used as non-fixing reference crop in order to determine plant-available soil nitrogen.

Plants were harvested at maturity (103 days after seedling emergence) and separated in seeds, pod walls and stem+leaves. Numbers of seeds and pods per pot were counted. Roots were harvested and carefully washed free of soil. After dry matter determination (80° C for 20 h) samples were ground and total N determined using a semi-micro Kjeldahl procedure including nitrate [2]. ¹⁵N-analysis was carried out on ammonium originating from the Kjeldahl digests using the modified Dumas combustion [3] for converting ammonium to N₂ gas and mass spectrometry for determining the ¹⁵N:¹⁴N ratios of the gas samples. A value of 0.366 was used for calculating ¹⁵N atom % excess.

The amounts of N derived from fertilizer, soil and symbiotic nitrogen fixation were estimated using the 'A'-value concept [4, 5] and barley as reference crop. Estimates were corrected for seed-borne nitrogen (Ndfk). A seed of pea or barley contained 10 and 1 mg N, respectively.

The amount of nitrogen derived from the fertilizer (Ndff) was calculated as:

$$\text{Ndff} = \frac{\text{atom \% } ^{15}\text{N excess, plant}}{\text{atom \% } ^{15}\text{N excess, fertilizer}} \times \text{total N} \quad (1)$$

The barley crop had accumulated 2.21 g N/pot at maturity of which 80.4% was derived from the fertilizer N. This corresponded to a fertilizer recovery of 74.3%. The 'A'-value was calculated as:

$$'A'\text{-value} = \text{Ndfs} \times 100 / \% \text{Fr} \quad (2)$$

where Ndfs= N derived from the soil and

%Fr= % of fertilizer N recovered.

Assuming that the same amount of soil N was available for pea and barley, the 'A'-value (0.55 g N/pot) and the respective fertilizer N recoveries in pea were used for estimating the amount of N derived from soil. Nitrogen fixation could then be calculated as :

$$\text{N}_2 \text{ fixation} = \text{Total N} - \text{Ndff} - \text{Ndfs} - \text{Ndfk} \quad (3)$$

An analysis of variance was carried out on the data. LSD (P=0.05) was used to compare means if F-tests showed significant treatment effects.

Results

Dry matter production and N content

N-fertilization with nitrate at sowing ("starter N") significantly increased the dry weight of both vegetative plant parts and roots (Table 1). The seed yield was also increased, but not significantly however (Table 1). "Starter N" at rates of 0.3 and 0.6 g N/pot significantly increased the mean number of seeds per pod by about 10%, but neither the number of pods per plant nor the 1000-seed weight were influenced (data not shown). Late and split supply significantly increased the dry weight of all plant parts (Table 1). The seed yield was increased about 30% by late and split supply, this was due mainly to significantly more (12-13%) pods per plant (data not shown).

The average N-concentrations in dry matter of seeds, pod walls, stem+leaves and roots were 3.48, 0.51, 1.23 and 2.42 %N, respectively. Fertilization with nitrate generally decreased, however not significantly, the N-concentrations in the different plant parts, whereas N supplied at the flat

Table 1. Dry-matter production (g/pot) of peas as influenced by rate and time of nitrate N supply.

N treatment		Plant part		
Time of supply	Rate g N/pot	Root	Stem + leaves*	Seeds
Sowing	0	4.5	41.2	66.5
Sowing	0.3	5.4	50.3	75.1
Sowing	0.6	6.5	54.3	74.7
Sowing	1.2	8.4	54.6	72.2
Sowing	2.4	6.0	44.8	64.2
First bloom	1.2	5.4	54.9	81.1
Flat pod	1.2	6.8	61.2	87.1
Flat pod	2.4	7.7	57.6	87.6
Split**	3 x 0.8	7.6	61.1	87.3
LSD(P=0.05)		1.6	10.9	12.8

* Pod walls included.

** N supplied at sowing, at first bloom and at the flat pod growth stage.

pod growth stage or as split applications increased the seed N and total N content significantly, compared with unfertilized pea (Fig. 1).

Origin of nitrogen

The relative contributions from the different N sources to total N are shown in Table 2 and estimates of symbiotic nitrogen fixation per pot in Fig. 2. In the peas that were not supplied with ^{15}N labelled fertilizer N the proportion of total N which was derived from the soil (Nd_{fs}) was estimated to 0.39 g N/pot from the linear relationship between root dry weight and estimates of Nd_{fs} from peas which received ^{15}N labelled fertilizer N. This value was also used for estimating nitrogen fixation in peas supplied with N-fertilizer at time of first bloom or during early reproductive development, since it would have been erroneous to use the 'A'-value from barley, because the fertilizer N was available for peas during only part of the growth period. In the split application treatment nitrogen fixation was estimated according to the method described by Fried and Broeshart [5].

Larger amounts of N-fertilizer at sowing time decreased the relative contribution from nitrogen fixation; 0.3 and 0.6 g N/pot did not significantly reduce the amount of nitrogen fixed, but 2.4 g N/pot almost completely inhibited the N_2 fixation (Table 2, Fig. 2). The mean contribution to total N from the seed N store and soil N was 4 and 12%, respectively (Table 2).

Nitrate supplied at the early reproductive stages and as split applications decreased the nitrogen fixation relatively and absolutely (Fig. 2, Table 2). The split application had the strongest effect on nitrogen fixation. The fertilizer nitrate had the least affect when supplied at the flat pod growth stage (Fig. 2).

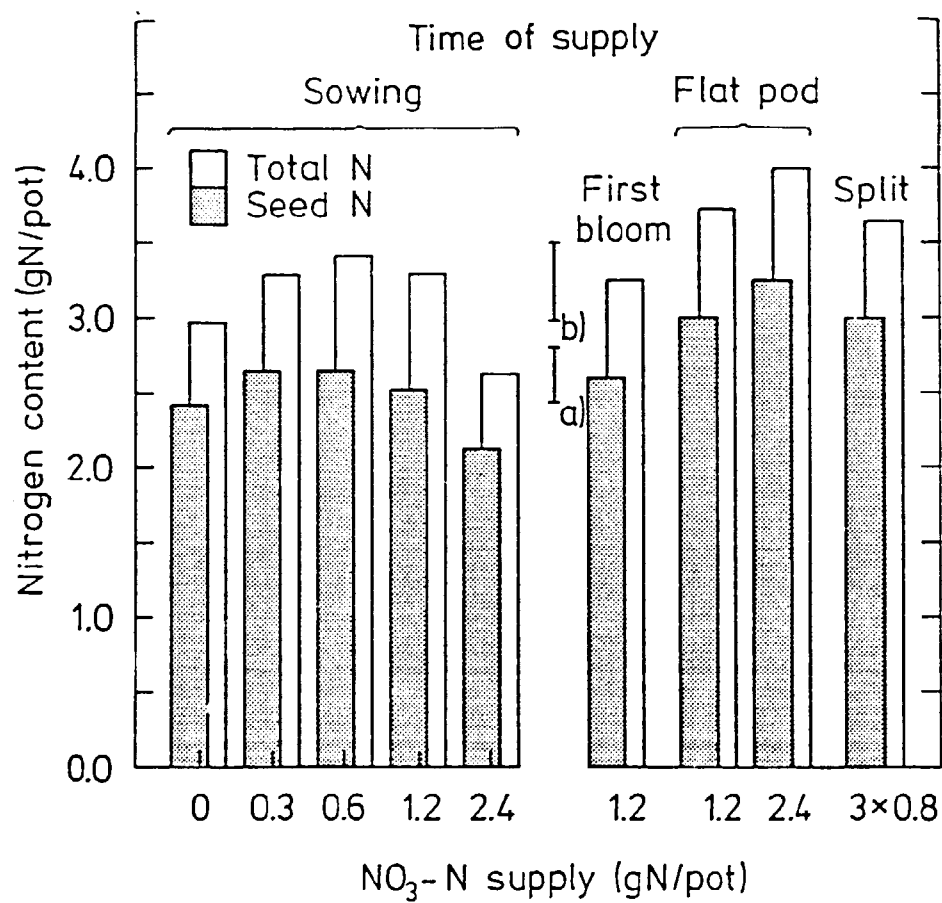


Fig 1. Seed and total N content in pea as influenced by rate and time of nitrate supply. a), LSD (P = 0.05) for seed N comparisons; b), LSD (P = 0.05) for total N comparisons.

Table 2. Estimates of the contributions (% of total N) from seed-borne, fertilizer, soil and symbiotically fixed nitrogen to total plant N in peas.

N treatment		Percent of total N derived from			
Time of supply	Rate g N/pot	Seed	Fertilizer	Soil	N ₂ fixation
Sowing	0	4.7	-	13.2*	82.1*
Sowing	0.3	4.2	6.6	12.3	76.9
Sowing	0.6	4.1	13.8	12.8	69.3
Sowing	1.2	4.2	30.7	14.2	50.9
Sowing	2.4	5.3	70.8	16.3	7.6
First bloom	1.2	4.3	29.7	11.6*	54.4*
Flat pod	1.2	3.7	29.6	9.2*	57.5*
Flat pod	2.4	3.5	52.5	9.8*	34.2*
Split	3 x 0.8	3.8	56.9	12.3	27.0

* See text for method of estimation.

Values in the table represent means of four replicates.

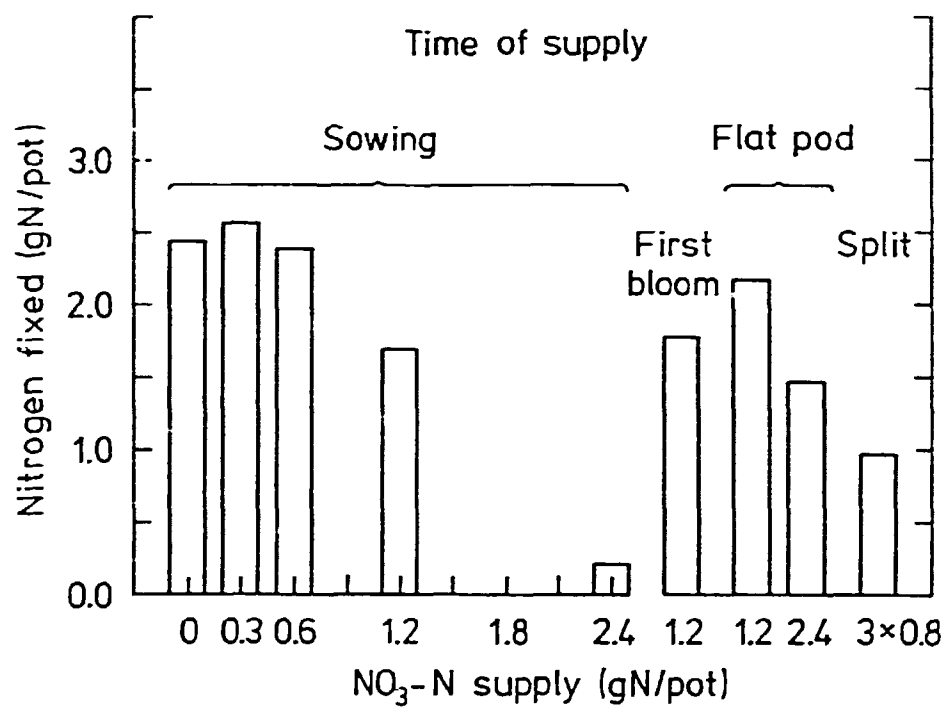


Fig. 2. Estimated nitrogen fixation in pea. (See text for methods of estimation).

Recovery of fertilizer N

The fertilizer N was taken up efficiently by the pea crop (Table 3). Increasing the supply of "starter N" resulted in an increasing recovery, and an increasing proportion of the fertilizer N was located in the seeds (Table 3). Delaying the time of supply reduced the content in roots and vegetative plant parts and increased the content in seeds resulting in an increase in the total recovery of fertilizer N in the crop (Table 3). When the nitrate was supplied at the flat pod growth stage 88% was recovered and about 90% of this fertilizer N was located in the seeds (Table 3).

Discussion

According to Pate [14] a low level of combined N in the soil solution will result in optimum pea yields, while still permitting symbiotic nitrogen fixation to contribute with the main part of total plant N. In the present experiment, where the soil contained 2.1 mg NO_3 and $\text{NH}_4\text{-N}$ /100 g dry weight, nitrogen fixation contributed with about 80% of total plant N in peas receiving no nitrate fertilizer. Small dressings of fertilizer nitrate at sowing stimulated vegetative growth, but seed dry matter production and N content were not significantly increased. This supports the general observation that "starter N" is not needed when peas are grown in soil with efficient strains of Rhizobium leguminosarum, and in which mineralized soil N can support the nitrogen nutrition of the plant until nodules are formed and functioning [14].

Moderate levels of "starter N" did not decrease N fixation significantly (Fig. 2). This confirms the observations by Oghoghorie and Pate [13], that nitrogen fixation by peas is rather tolerant to high levels of combined N. In the present experiment the fertilizer nitrate was placed 12 cm below the seed. This deep placement may have reduced the negative effect of nitrate on nodule formation and functioning,

Table 3. Recovery of fertilizer nitrate in different pea plant parts (mg N/pot) and the total recovery as percent of N supplied.

N treatment		Fertilizer N recovered in				Percent of fertilizer N recovered
Time of supply	Rate g N/pot	Root	Stem + leaves	Pod walls	Seed	
Sowing	0.3	23 ± 1	44 ± 8	5 ± 0.2	145 ± 3	72.7 ± 4.1
Sowing	0.6	40 ± 4	105 ± 9	12 ± 1	316 ± 11	79.0 ± 1.6
Sowing	1.2	79 ± 8	178 ± 10	32 ± 1	729 ± 16	85.0 ± 1.8
Sowing	2.4	71 ± 3	200 ± 6	47 ± 1	1572 ± 41	78.7 ± 1.9
First bloom	1.2	24 ± 3	84 ± 4	32 ± 3	817 ± 20	79.7 ± 1.5
Flat pod	1.2	23 ± 2	60 ± 7	26 ± 3	961 ± 22	89.1 ± 2.2
Flat pod	2.4	41 ± 1	127 ± 8	48 ± 7	1873 ± 32	87.1 ± 1.3
Split	3 x 0.8:					
sowing	0.8	48 ± 3	113 ± 5	16 ± 1	477 ± 5	81.8 ± 0.8
first bloom	0.8	14 ± 1	47 ± 2	23 ± 2	600 ± 9	85.5 ± 1.2
flat pod	0.8	13 ± 1	38 ± 3	18 ± 2	661 ± 14	91.2 ± 2.1
total		75 -	198 -	57 -	1738 -	86.2 -

Values in the table are means (± SE) of four replicates.

since Harper and Cooper [7] found that uniform incorporation of ammonium nitrate in the soil impaired nodule development more than deep application of the fertilizer. Higher levels of nitrate N, however, significantly reduced the symbiotic nitrogen fixation, irrespective of the relatively deep placement of the fertilizer N in the present experiment.

The late supply of nitrate N, especially at the flat pod growth stage, and the split supply at sowing, first bloom and at the flat pod growth stage significantly increased the yield of seed dry matter and nitrogen as was found by Andersen et al. [1]. Whether or not this effect can be obtained in the field as well has yet to be studied.

Schilling et al. [17] found that the nitrogen fixation of peas, that were supplied with ammonium nitrate after flowering, was reduced only about 16%. In the present experiment the N_2 fixation was reduced about 11% compared with the unfertilized control, when peas received 1.2 g NO_3 -N/pot at the flat pod growth stage. When nitrate was supplied at the time of first bloom or as split applications nitrogen fixation was decreased considerably. This indicates that the late N supply should first be applied after termination of flowering, in order to obtain maximum nitrogen fixation.

Since the rate of nitrogen fixation in peas is decreased during reproductive development [9], it is interesting to note that the pea plants had the capacity for uptake and assimilation of nitrate during these growth stages. In general, the peas took up the nitrate N supplied very efficiently. The recovery of 2.4 g N/pot supplied at sowing was higher in peas than in barley, 79 and 74% respectively. Within moderate levels the higher the nitrate N supply at sowing, the higher was the recovery of the fertilizer N (Table 3). Presumably, this was due to immobilization of the applied N in soil organic matter. At the lower fertilizer N levels immobilization will affect the percent N recovered more, since the amount of organic material available for immobilization would be the same. However it is noteworthy that the root

dry matter production was correlated to the recovery of fertilizer N. (Table 1, Table 3).

It is evident from the treatments with supply of N at different times, that the recovery of N-fertilizer was higher the later it was supplied, and the proportion that was located in the seeds was also higher. Schilling et al. [17] (pea) and Andersen et al. [1] (pea) and Richards and Soper [15] (field bean) also found, that the later the N-fertilizer was supplied, the higher was the recovery. Presumably, this was because the later the N was supplied the more developed was the root system, the quicker was the uptake and the less was immobilization of the fertilizer N. In this context it must be considered that this experiment was carried out in pot with optimal watering. In the field the uptake of soil applied nitrate N during the reproductive growth stages may be limited by low soil moisture.

The observation that pea take up and assimilate nitrate very efficiently during reproductive development is in contrast to the observation and statement by Rigaud [16] that legumes generally do not respond to N-fertilization during reproductive growth. This follows because the absorption of nitrate and its reduction are restrained by natural senescent processes occurring in the plant at these growth stages [16]. The capacity for nitrate uptake and metabolism during reproductive development in pea, might be because the reduction of NO_3 and N_2 are performed at different sites. It is assumed that pea reduces the main part of the nitrate taken up in the root system [14], but when higher amounts of nitrate N become available, the root nitrate reductase is saturated, and nitrate is transported to the shoot and reduced there [13]. According to Schrader and Thomas [18] the reduction of nitrate in green plant parts in light may be only half as energy demanding as in the roots. The transport to and reduction of nitrate in the shoot may thus be the reason why pea can take up and assimilate nitrate during reproductive growth stages, where the rate of nitrogen fixa-

tion normally is decreased, due to carbohydrate deprivation of the root nodules.

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The Influence of Seed-borne N in ^{15}N Isotope Dilution Studies with Legumes

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The distribution of seed-borne N in shoot and root of pea and field bean was studied using three methods: 1) determination of the N content in shoot and root of plants grown in sand culture without other N sources, 2) ^{15}N isotope dilution in plants grown in *Rhizobium*-free medium supplied with ^{15}N -labelled nitrate and 3) determination of the ^{15}N -enrichment in shoot and root of plants in which the seed-borne N was labelled with ^{15}N . The results from the three methods were concordant, showing that from 44 to 50% of the seed-borne N in the two species was located in above-ground plant parts. The effect of corrections for seed-borne N in studies of nitrogen fixation in legumes is discussed. *Key-words*: A-value, barley, field bean, nitrogen fixation, non-fixing reference, pea.

INTRODUCTION

Legumes may obtain their nitrogen both from symbiotic N_2 fixation and assimilation of soil and fertilizer nitrogen. In order to study the relative importance of these processes under different growth conditions, one should be able to measure the contribution of each of these N sources in the supply of nitrogen to plants.

Use of ^{15}N -labelled nitrogen fertilizers enables us to determine in a non-fixing plant the nitrogen derived from the applied fertilizers and from the soil, respectively. The per cent of plant nitrogen deriving from the ^{15}N -labelled fertilizer (%Ndff) is calculated as:

$$\% \text{Ndff} = \frac{\text{atom } \% \text{ } ^{15}\text{N} \text{ excess, plant}}{\text{atom } \% \text{ } ^{15}\text{N} \text{ excess, fertilizer}} \times 100 \quad (1)$$

and the proportion from the soil by the difference calculation: $100 - \% \text{Ndff}$.

If it is assumed that the plant assimilates the soil and fertilizer nitrogen in proportion to the respective quantities available, we can estimate the 'A'-value (Fried & Dean, 1952), which is the quantity of soil nitrogen participating in the supply of nitrogen to the plant.

$$\text{'A'-value} = \frac{100 - \% \text{Ndff}}{\% \text{Ndff}} \times \text{fertilizer N} \quad (2)$$

A procedure for measurement of the amount of nitrogen fixed by a legume crop was presented by Fried & Broeshart (1975). This method involves simultaneous determination of the 'A'-values for the legume and a non-fixing reference crop. The amount of symbiotically fixed nitrogen is then calculated by multiplying the difference in 'A'-value between the legume and the non-fixing reference crop with the per cent utilization of fertilizer nitrogen by the legume crop. A simplified procedure for the estimation of N_2 fixation, which can be used when identical amounts of ^{15}N labelled N-fertilizer are applied to legume and reference crop was presented by Fried & Middelboe (1977)

$$N_2 \text{ fixed} = \left[1 - \frac{\text{atom \% } ^{15}\text{N excess, legume}}{\text{atom \% } ^{15}\text{N excess, reference}} \right] \times \text{total } N_{\text{legume}} \quad (3)$$

In this procedure it is assumed that the proportions of nitrogen taken up from soil and fertilizer is the same for the legume and the reference crop. The reference crop may be a non-fixing legume or a non-legume.

Besides from soil, fertilizer and symbiotically fixed N, plants derive some of their nitrogen from seed-borne N. In plant species with different amounts of seed-borne N, this nitrogen may dilute the ¹⁵N taken up from the growth medium to different extent and invalidate the calculations. In our current investigations (Jensen, 1986) we used barley as a reference crop in estimating symbiotic N₂ fixation in pea and field bean. However, the seeds of pea and field bean contain much more nitrogen than barley seeds. This difference in N content is included as symbiotically fixed nitrogen if estimates are not corrected. The present investigations were carried out in order to estimate the amount of seed-borne N recovered in the shoot and root of pea and field bean and to evaluate the effect of corrections for seed-borne N on estimates of N₂ fixation.

MATERIALS AND METHODS

The experiments were carried out in green-houses during the summertime. The following crop cultivars were used: pea (*Pisum sativum* L.) cv. 'Bodil', field bean (*Vicia faba* L.) cv. 'Diana' and barley (*Hordeum vulgare* L.) cv. 'Nery'.

Experiment 1

Pea, field bean and barley were grown in sand cultures without *Rhizobium leguminosarum* and without combined N for two weeks after emergence. Four replicates of each plant species were arranged in a randomized block design.

Experiment 2

Pea, field bean and barley were grown in soil sterilized by irradiation (4 Mrad) in order to obtain a growth medium free of *Rhizobium*. Eight plants of pea, field bean and barley were grown in pots containing 4.5 kg soil. Appropriate amounts of essential plant nutrients including 300 mg N pot⁻¹ as K¹⁵NO₃ (5.60 atom% ¹⁵N excess) were added. Pots were placed in a randomized complete block design with 4 replicates. Crops were harvested 4 weeks after seedling emergence. Visual inspection of the roots ensured that nodules were not formed.

Experiment 3

Pea seeds labelled with ¹⁵N were obtained from a pot experiment with ¹⁵N. Four intact pea pods containing 4 seeds each were selected for the experiment. Dry matter, Kjeldahl N, and the ¹⁵N/¹⁴N ratio was determined on a single seed from each pod. The mean seed N store was 11 mg N per seed with 0.133 (±6.0%) atom% ¹⁵N excess. It was assumed that all seeds within a single pod had the same ¹⁵N-enrichment. The remaining seeds were sown in pots containing 1.5 kg soil. The pots were placed in a randomized block design with four replicates. Plants were harvested 3 weeks after seedling emergence.

Dry matter and nitrogen determinations

The dry matter content of plant parts were determined by drying samples at 80°C in a well-aerated oven for 20 h. Samples were ground and total N determined by a semi-micro Kjeldahl method (Bremner & Mulvaney, 1982). The ¹⁵N/¹⁴N ratios of ammonium N

Table 1. Recovery of seed-borne N in top and root of pea, field bean and barley plants grown in *Rhizobium* and N-free medium

Values are means of four replicates (\pm SD)

Crop	Seed N store (mg N seed ⁻¹)	N content		Seed-N recovered	
		Top (mg N plant ⁻¹)	Root (mg N plant ⁻¹)	Top (%)	Root (%)
Pea	10.1	4.5 \pm 0.2	5.2 \pm 0.8	44	52
Field bean	18.4	8.8 \pm 0.3	9.0 \pm 0.2	48	49
Barley	0.7	0.32 \pm 0.01	0.36 \pm 0.01	45	52

derived from the Kjeldahl distillates were determined according to the methods described by Fiedler & Proksch (1975) by modified Dumas combustion and mass spectrometry.

RESULTS AND DISCUSSION

Distribution of seed-borne N in plants grown without an external N-source

The N content in top and root + seed remnants of pea, field bean and barley grown for two weeks after seedling emergence in *Rhizobium*- and N-free medium is shown in Table 1. About 95% of the seed-borne N was recovered and it was almost evenly distributed between shoot and root.

These plants were N-stressed since there were no other N-sources than the seed N store. The distribution of the seed N might change if plants are grown with a normal N-supply for a longer growth period. The following two experiments were designed to study the distribution of seed-borne N in grain legumes grown with other sources of N.

Distribution of seed-borne N in N-fertilized plants

Results from this experiment where plants were grown in *Rhizobium*-free soil, but supplied with ¹⁵N-labelled nitrogen are shown in Table 2. The atom% ¹⁵N excess varies considerably among the plant species and between root and shoot (Table 2). This might lead to the conclusion that soil-N differs in availability to the different plant species. However, it is reasonable to assume that the legumes and barley assimilate fertilizer and soil-N in the same proportions when grown in a pot experiment with small soil volumes as in the

Table 2. Seed N store of seeds sown and N content and atom% ¹⁵N excess in top and root of plants four weeks after seedling emergence

Plants were grown in *Rhizobium*-free soil and supplied with ¹⁵N-labelled NO₃-N. Values are means of three replicates (\pm SD)

Crop	Seed N store (mg N pot ⁻¹)	N content		Atom% ¹⁵ N excess	
		Top (mg N pot ⁻¹)	Root (mg N pot ⁻¹)	Top (%)	Root (%)
Pea	71	259 \pm 27	69 \pm 11	3.08 \pm 0.11	2.47 \pm 0.09
Field bean	147	277 \pm 24	136 \pm 5	2.59 \pm 0.10	2.15 \pm 0.05
Barley	5	239 \pm 2	52 \pm 10	3.49 \pm 0.11	2.64 \pm 0.04

Table 3. Nitrogen derived from the seed N store in top and root of pea and field bean
Calculated from equation 3 with corrected atom % ¹⁵N excess of barley plant parts

Legume	N derived from seed		Seed-N recovered	
	Top (mg N pot ⁻¹)	Root (mg N pot ⁻¹)	Top (%)	Root (%)
Pea	32	8	45	11
Field bean	73	31	50	21

present experiment. When comparing the difference in the seed N store between pea, field bean and barley (Table 1), the apparent difference in atom % ¹⁵N excess might be explained by N contributions from the seed N store diluting the ¹⁵N-labelled nitrogen taken up from the soils to different extent.

Equation 3 can be used to calculate the contributions from the seed N store (seed N equal to N₂ fixed) to different plant parts of the legumes, provided the plants are grown in *Rhizobium*-free soil and assimilate soil and fertilizer N in the same proportions as the barley crop. It is, however, necessary to assume that the seed-borne N of barley is distributed as found in Exp. 1 (Table 1). The atom % ¹⁵N excess of barley plant parts is then corrected by the following equation:

$$\text{atom \% } ^{15}\text{N excess}_{\text{corr.}} = \frac{N \times \text{atom \% } ^{15}\text{N excess}}{N - N_{\text{seed}}} \quad (4)$$

where N = N content in top or root and N_{seed} = N in top or root derived from the seed N store.

The corrected atom % ¹⁵N excess of barley top and root were 3.52 and 2.78, respectively. Then the amount of seed-borne N in shoot and root of pea and field bean is calculated from equation 3 using the corrected atom % ¹⁵N excess of barley. The amount of seed-borne N recovered in the shoot (Table 3) accords well with the recovery of seed-borne N in the shoot found in Exp. 1 (Table 1). The amount of seed-borne N recovered in the root was lower than in Exp. 1 (Table 1). This might be due to a lower recovery of root material in soil culture compared to sand culture.

If the per cent recovery of seed-borne N found in Exp. 1, is used to correct atom % ¹⁵N excess of plant parts in Exp. 2 by means of equation 4, the corrected atom % ¹⁵N excess of pea and field bean tops were found to be 3.50 and 3.40, respectively, compared to 3.52 in barley. This supports the assumption that the three crops took up fertilizer and soil N in the same proportion.

Distribution of seed-borne N in N₂-fixing plants

In this experiment the distribution of the seed-borne N in pea plants assimilating soil nitrogen and fixing N₂ symbiotically was evaluated by means of ¹⁵N-labelled seeds. Plants grown for three weeks after seedling emergence contained 12 and 10 mg N per plant in top and root, respectively. The atom % ¹⁵N excess in top and root were 0.059 and 0.043 respectively. The amount of N derived from seed-borne N in pea top and roots were calculated using equation 1 with the atom % ¹⁵N excess of the seed N store (0.133) as the denominator. Five and 3 mg seed N per plant, corresponding to 48 and 29% of the seed N store, was recovered in top and root, respectively. The results are in accordance with the recovery of seed-borne N in peas in Experiments 1 and 2, indicating that about 50% of the seed N store is recovered in the plant tops.

Effect of correction for seed-borne N on estimates of nitrogen fixation

Studies of the utility of different reference crops in the ^{15}N fertilizer dilution technique (Wagner & Zapata, 1982; Witty, 1983; Fried et al., 1983) have often shown that species differ in ^{15}N -enrichment and consequently the %Ndff and 'A'-values differ. There may be different reasons for this (Witty, 1983; Fried et al., 1983), but these differences may to some extent be attributed to seed-borne N in the plants, diluting the ^{15}N to different extent.

Calculating 'A'-values from %Ndff of shoots of crops which were derived from uncorrected atom % ^{15}N excess (Table 2) by means of equation 2, gave the following 'A'-values: 245, 347 and 181 mg N for pea, field bean and barley, respectively. Calculation of 'A'-values using %Ndff derived from corrected atom % ^{15}N excess, which were identical for the three species, would according to equation 2 result in similar 'A'-values being: 177 mg N.

Therefore it is important to correct estimates of symbiotic nitrogen fixation for seed-borne N. Corrections for seed-borne N should especially be considered when the ^{15}N fertilizer dilution technique is used to compare symbiotic nitrogen fixation in grain legumes species and varieties with large differences in the seed N store at the early growth stages. Omitting correction may e.g. lead to 10 to 15% overestimation of N_2 fixation in pea.

These results indicate that determination of the distribution of seed-borne N in plants grown a few weeks with the seed N store as the only N source, are sufficiently for obtaining a reliable estimate of the seed N distribution.

When the 'A'-value concept is used for estimating nitrogen fixation it is easy to correct estimates of N_2 fixation by subtracting the amount of seed-borne N located in the respective plant parts. When the principle proposed by Fried and Middelboe (1977) (equation 3) is used it is necessary to correct the atom % ^{15}N excess of the legume and the reference crop plant parts by means of equation 4.

CONCLUSION

Studies of the distribution of seed-borne N in pea and field bean showed that about 50% of this N was recovered in the above-ground plant parts. The amount recovered in root was more variable and differed among experiments.

When another species, than the legume in question, is used as a reference crop in the ^{15}N fertilizer dilution technique it is necessary to correct estimates of N_2 fixation for seed-borne N. Otherwise this N will be included in the estimate of nitrogen fixed symbiotically.

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<p>Abstract (Max. 2000 char.)</p> <p>Symbiotic nitrogen fixation and nitrate uptake by pea plants (<i>Pisum sativum</i> L.) were studied in field and pot experiments using the ^{15}N isotope dilution technique and spring barley as a non-fixing reference crop. Barley, although not ideal, seemed to be a suitable reference for pea in the ^{15}N-technique. Maximum N_2 fixation activity of 10 kg N fixed per ha per day was reached around the flat pod growth stage, and the activity decreased rapidly during pod-filling. The pea crop fixed between 100 and 250 kg N ha^{-1}, corresponding to 45 to 80% of total crop N. The amount of symbiotically fixed N_2 depended on the climatic conditions in the experimental year, the level of soil mineral N and the pea cultivar. Field-grown pea recovered 60 to 70% of the N-fertilizer supplied. The supply of 50 kg $\text{NO}_3\text{-N ha}^{-1}$ inhibited the N_2 fixation approximately 15%. Small amounts of fertilizer N, supplied at sowing (starter-N) slightly stimulated the vegetative growth of pea, but the yields of seed dry matter and protein were not significantly influenced.</p> <p>In the present field experiments the environmental conditions, especially the distribution of rainfall during the growth season, seemed to be more important in determining the protein and dry matter yield of the dry pea crop than the ability of pea to fix nitrogen symbiotically. However, fertilizer N supplied to pot-grown pea plants at the flat pod growth stage or as split applications significantly increased the yield of seed dry matter and protein.</p>	
<p>Descriptors - INIS</p> <p>BACTERIA; CROPS; FERTILIZERS; ISOTOPE DILUTION; NITRATES; NITROGEN FIXATION; NITROGEN 15; PEAS; SYMBIOSIS; UPTAKE</p> <p>Available on request from Riso Library, Riso National Laboratory, (Riso Bibliotek, Forskningscenter Riso), P.O. Box 40, DK-4000 Roskilde, Denmark. Telephone 02 37 12 12, ext. 2202. Telex: 43116, Telefax: 02 35 05 00</p>	